

Nephrinuria in Diabetic Nephropathy of Type 1 Diabetes

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Diabetic nephropathy is the leading cause of end-stage renal disease. Because early diagnosis and treatment may prevent the complication, new tools for an early detection are needed. One of the key components of the glomerular filtration slit spanning between neighboring podocytes is nephrin. Its expression is altered in experimental models of diabetes and also in various human proteinuric diseases, including diabetes. We studied whether type 1 diabetic patients with or without nephropathy exhibit immunoreactive nephrin in the urine, reflecting early damage of the filtration barrier. Diabetic patients with normoalbuminuria ($n = 40$), with microalbuminuria ($n = 41$), and with macroalbuminuria ($n = 39$) and patients previously normoalbuminuric but now testing positive for microalbuminuria (newMicro, $n = 39$) were screened for nephrinuria with Western blotting using two affinity-purified anti-nephrin antibodies. Nondiabetic healthy subjects ($n = 29$) were also studied. Nephrinuria was present in 30% of normoalbuminuric, 17% of microalbuminuric, 28% of macroalbuminuric, and 28% of newMicro patients. Of female patients, 35% were nephrinuric compared with only 19% of male patients ($P = 0.02$). None of the control subjects was nephrinuric. In conclusion, glomerular filtration barrier may be affected in one-third of diabetic patients manifesting as early nephrinuria. Nephrinuria may have prognostic value and become a marker of susceptibility for kidney complications in diabetes. *Diabetes* 52:2969–2974, 2003

Diabetes is a rapidly increasing health care problem all over the world, showing a fivefold increase in the prevalence figures during the past 15 years. In parallel, one of the main diabetic complications, nephropathy, is also increasing. Diabetic nephropathy is a devastating chronic event that is characterized by persistent proteinuria, elevated arterial blood pressure, and decline in renal function. Overt nephropathy is diagnosed when the albumin excretion rate

(AER) persistently exceeds 300 mg in a 24-h urine collection (1).

The decline in renal function and glomerular filtration rate arises from damage to the glomerular filtration barrier. This is accompanied by deterioration of tubular reabsorption, increase of circulating creatinine, and morphologically by accumulation of extracellular matrix and thickening of the basement membrane within the glomeruli (2,3). Ultimately, diabetic nephropathy leads to excessive scarring and a nonfunctional end-stage kidney requiring dialysis and transplantation therapies. Microalbuminuria, which is the earliest detectable marker of nephropathy, precedes overt nephropathy and is defined as a persistent AER ranging from 30 to 300 mg/24 h. It is also a strong predictor of subsequent nephropathy in type 1 diabetes (4,5). At the time of microalbuminuria, the glomeruli already demonstrate advanced glomerulopathy (6).

The filtration barrier of a glomerulus comprises capillary endothelium; glomerular basement membrane; and specialized visceral epithelial cells, the podocytes, which are located on the outer surface of the barrier (7). Podocytes are important for the maintenance of the dynamic functional filtration barrier (8), and recent data suggest that the podocyte number may be reduced in the glomeruli of both type 1 and type 2 diabetic patients (9,10). Furthermore, longitudinal data provide evidence for an association of podocyte loss and increasing AER in type 1 diabetes (11). It is presumable that there are earlier signs of perturbed podocyte metabolism before podocyte loss. Nephrin, a transmembrane protein of the immunoglobulin superfamily, seems to be a key molecular component of the filtration slit diaphragm between neighboring podocytes (12–14). Its major mutations lead to the massively proteinuric disease congenital nephrotic syndrome of the Finnish type (12,15). Pancreatic β -cells also express nephrin (16). Various experimental models of diabetes and hypertension show alterations in nephrin mRNA or protein levels in the glomeruli (17,18). In line with this, nephrin protein expression seems to be altered in various human proteinuric kidney diseases (19–21).

Given the rapidly increasing burden of diabetes and its complications, it is important to establish earlier predictive markers than microalbuminuria to allow early detection and early aggressive treatment. At the moment, no such markers are available. This study assessed the presence of immunoreactive nephrin in the urine of type 1 diabetic patients with or without nephropathy, reflecting damage of the filtration barrier.

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AER, albumin excretion rate; lnAER, log_e transformation of AER; RAS, renin-angiotensin system.

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TABLE 1
Clinical characteristics of the 159 type 1 diabetic patients in the study

	Normo	NewMicro	Micro	Macro
<i>n</i> (M/F)	40 (21/19)	39 (23/16)	41 (23/18)	39 (23/16)
Age (years)	42.1 ± 1.7	32.1 ± 1.9	30.8 ± 1.2	36.9 ± 1.5
Duration of diabetes (years)	28.5 ± 1.5	19.9 ± 1.8	19.0 ± 0.7	23.8 ± 1.1
BMI (kg/m ²)	25.7 ± 0.5	24.5 ± 0.5	25.9 ± 0.4	25.5 ± 0.7
Waist-to-hip ratio	0.859 ± 0.014	0.859 ± 0.013	0.874 ± 0.011	0.887 ± 0.016
Systolic blood pressure (mmHg)	131 ± 2	134 ± 3	133 ± 3	142 ± 3
Diastolic blood pressure (mmHg)	78 ± 1	79 ± 1	83 ± 2	86 ± 1
ACE inhibitor/AT2 blocker (%)	5	10	63	77
Non-RAS drug (%)	0	5	2	18
Coronary heart disease (%)	5	0	0	5
Current smoking (%)	21	31	40	36
HbA _{1c} (%)	8.4 ± 0.2	9.3 ± 0.3	9.2 ± 0.2	8.9 ± 0.3
Serum total cholesterol (mmol/l)	4.83 ± 0.12	4.71 ± 0.16	4.93 ± 0.13	5.50 ± 0.15
Serum HDL cholesterol (mmol/l)	1.61 ± 0.06	1.56 ± 0.08	1.56 ± 0.08	1.38 ± 0.08
Serum triglycerides (mmol/l)	1.13 ± 0.07	1.23 ± 0.14	1.10 ± 0.09	1.60 ± 0.12
Serum creatinine (μmol/l)	84 (68–114)	84 (47–120)	85 (55–132)	110 (66–600)
AER (mg/24 h)	8 (2–24)	42 (30–255)	70 (30–380)*	740 (70–6,069)†

Data are means ± SE, median (range), or *n* (%). *One previously microalbuminuric patient had AER over the range of microalbuminuria at the time of investigation (380 mg/24 h). †Some patients with previously abnormal AER had responded to antihypertensive treatment and showed a regression of AER at the time of investigation.

RESEARCH DESIGN AND METHODS

Subjects. We obtained 24-h urine samples from 159 type 1 diabetic patients from the ongoing Finnish Diabetic Nephropathy Study (FinnDiane): 40 normoalbuminuric (Normo), 41 microalbuminuric (Micro), and 39 macroalbuminuric (Macro) patients. In addition, we obtained urine samples from a group of patients (*n* = 39) designated as new microalbuminuria (newMicro). These patients had previously been normoalbuminuric, but our sample was the first showing microalbuminuria. The average time between the previous normoalbuminuric sample and our study sample was 6.2 months (SD 5.8). Similarly, the Macro patients had a recent onset (<2 years) of the diabetic kidney complication. Microalbuminuria was otherwise defined as a 24-h urinary AER of 30–300 mg in two of three consecutive 24-h urine collections, macroalbuminuria as AER >300 mg/24 h, and normoalbuminuria as a persistent AER <30 mg/24 h. The FinnDiane study is a multicenter, nationwide study that includes all adult type 1 diabetic patients from 90 Finnish hospitals and primary care health centers with a target to study 25% of the Finnish type 1 diabetic population, namely 8,000 patients. Morning urine collections from 29 (18 female and 11 male) healthy nondiabetic laboratory personnel were obtained for control samples. Albumin-to-creatinine ratios of the samples were determined in the central laboratory of Helsinki University Hospital using standard protocols. All control subjects were normoalbuminuric (0.56 ± 1.07 [mean ± SD]) except for one female, who had low microalbuminuric value 5.3 mg/mmol (microalbuminuric range for women 3.5–35 mg/mmol). Every patient gave a written informed consent, and the study was approved by the local ethics committee.

Sample preparation. AER was measured using commercial radioimmunoassay (Pharmacia, Uppsala, Sweden; 2 mg/l detection limit and interassay coefficient of variance 5%) and total urinary protein concentration with colorimetric RC CD Protein Assay (Bio-Rad Laboratories, Hercules, CA) using microfuge tube assay protocol according to the manufacturer's instructions. Sample volumes corresponding to 30 μg of total protein were precipitated with 10% (wt/vol) trichloroacetic acid in PBS on ice for 30 min and then centrifuged for 10 min at 13,100g at 4°C, and the pellets were washed twice with ice-cold acetone. The samples were air-dried and dissolved in Laemmli buffer (62.5 mmol/l Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, and 0.05% bromophenol blue) followed by heating at 95°C for 5 min. Because the relative proportion of albumin from total urinary proteins was so much higher in the Micro and Macro groups, there was a possibility that the negative patients were false negatives. Therefore, we also analyzed a corrected urine sample from each negative patient of the Micro and Macro groups. A larger urine volume corresponding to 30 μg of other proteins than albumin (other proteins = amount of total protein – amount of albumin) was precipitated as above. Indeed, 9 of the 11 nephritic samples in the Macro group were found by analyzing a corrected sample, whereas no new positives were found in the Micro group after correction. Five samples from the Micro group had run out and could not be analyzed again as the corrected sample. **SDS-PAGE and Western blotting.** The proteins were run through reducing 10% polyacrylamide gels in the Protean Mini-gel electrophoresis system

(Bio-Rad Laboratories) and then transferred to nitrocellulose filters (Amersham Biosciences, Buckinghamshire, U.K.). After blocking for 2 h at room temperature with 3% nonfat dried milk (Valio, Helsinki, Finland) in PBS, the filters were incubated with antibodies Aff338 (1:5) and Aff380 (1:5) in PBS containing 1% nonfat dried milk and 0.02% sodium azide for 1 h at room temperature. The filters were then washed several times in PBS containing 0.2% Tween 20, further incubated with peroxidase-conjugated affinity-purified goat anti-rabbit IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA; 1:40,000) for 1 h at room temperature, and washed as above. The bound antibodies were detected with Super Signal enhanced chemiluminescence substrate (Pierce, Rockford, IL). The Aff338 and Aff380 rabbit polyclonal antibodies were originally raised against the major splicing variant designated as nephrin α using a recombinant fusion protein α-435 as the antigen as described earlier (16). It carries parts of the intra- and extracellular regions of nephrin (amino acids 1031–1055 and 1096–1215), thus lacking the exon 24. Presence of the characteristic protein bands visible with both antibodies in Western blots was regarded as positive for nephrin.

Immunofluorescence. Normal kidney tissue was obtained from cadaver kidneys taken for transplantation but not grafted because of vascular anatomical abnormalities (Department of Surgery, University of Helsinki, Helsinki, Finland) in accordance with the principles of the Declaration of Helsinki. Five-micrometer-thick human cortex cryostat sections were air-dried and then fixed in ice-cold acetone for 5 min before washes in PBS. The sections were then incubated with Aff338 (1:1), Aff380 (1:1), rabbit anti-human glucagon antibody (Zymed, San Francisco, CA; 1:50) or no primary antibody in 1% normal goat serum in PBS overnight at 6°C. After washes in PBS, the sections were incubated with tetramethylrhodamine-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch; 1:200) for 30 min at room temperature. After washes in PBS, the slides were covered with Imm-mount mounting medium (Shandon, Pittsburgh, PA). Microscopy was performed with an Olympus BX50 microscope (Olympus Optical, Tokyo, Japan) equipped with a cooled digital camera (Hamamatsu Photonics, Hamamatsu City, Japan). Openlab 2.2.3 (Improvision, Coventry, U.K.) and Adobe Photoshop (Adobe Systems, San Jose, CA) software was used for image documentation.

Statistical analysis. Data are expressed as mean ± SE unless otherwise stated. Differences between group means were tested with ANOVA, Mann-Whitney, Kruskal-Wallis, or Pearson χ^2 tests when appropriate. The correlation between AER and urinary albumin-to-total protein ratio was tested with linear regression analysis after log_e transformation of AER (lnAER). All analyses were performed using the BMDP statistical package (BMDP Statistical Software, Los Angeles, CA). *P* < 0.05 was considered significant.

RESULTS

Table 1 presents the clinical characteristics of the diabetic patients. Of Normo type 1 diabetic patients, 30% exhibited nephrin-specific protein bands in the urine, and the occur-

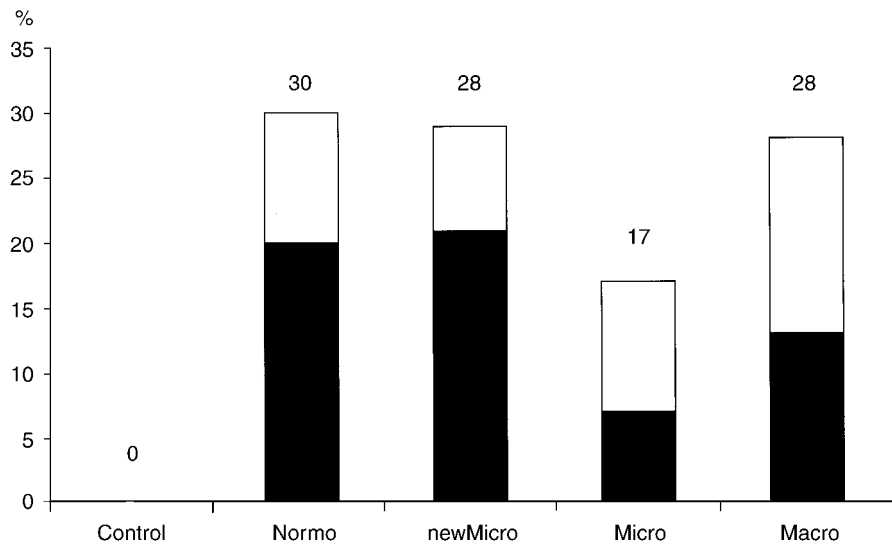


FIG. 1. Percentage of nephrinuric type 1 diabetic patients in the test groups. □, men; ■, women.

rence rate of nephrinuria was closely the same for the newMicro (28%) and Macro (28%; Fig. 1). All healthy nondiabetic control subjects were non-nephrinuric. The occurrence of nephrinuria was lowest in the Micro group, but the difference was not statistically significant. It is interesting that of all of the diabetic female patients, 35% were nephrinuric compared with only 19% of the male patients ($P = 0.02$). Nephrinuric and non-nephrinuric patients did not differ from each other with respect to any of the measured variables listed in Table 1 (data not shown). It is interesting that urinary albumin-to-total protein ratio increased along with severity of the kidney disease (Normo 0.03, newMicro 0.09, Micro 0.28, and Macro 0.49; $P < 0.0001$). Accordingly, there was a strong correlation between lnAER and urinary albumin-to-total protein ratio (Pearson, $r = 0.8684$, $P < 0.0001$, $n = 159$; Fig. 2).

The urine analyses with the Western blotting method using two affinity-purified nephrin antibodies revealed protein bands typically for molecular weights of 18, 32, 40, 60, or 75 kDa (Fig. 3). The antibodies recognized the typical full-length nephrin (185 kDa) in human glomerular lysate as well as the recombinant fusion protein α -435 that was used as a positive control in the blots. Immunofluorescence microscopy revealed an epithelial-type glomeru-

lar staining typical for nephrin with both Aff338 and Aff380 antibodies (Fig. 4). Control stainings with an irrelevant anti-human glucagon antibody and secondary antibody alone without any primary antibody showed negligible reactivity.

DISCUSSION

These findings show for the first time that fragments of the podocyte protein nephrin are found in the urine of type 1 diabetic patients. One-third of the patients were nephrinuric despite normal AER, whereas none of the control subjects demonstrated nephrinuria, suggesting that the glomerular filtration barrier and particularly the interpodocyte bridge may be affected by diabetes.

We used affinity-purified antibodies in our analyses, revealing the typical 185-kDa protein band of nephrin in Western blot of isolated glomeruli and glomerular immunofluorescence staining typical for nephrin. Unlike our earlier results in experimental diabetic nephropathy (22), we did not find the full-length nephrin in the urine of the study patients; thus, the nephrin released from the podocytes is most likely split into typical fragments during the passage through the nephrons, especially in the tubular

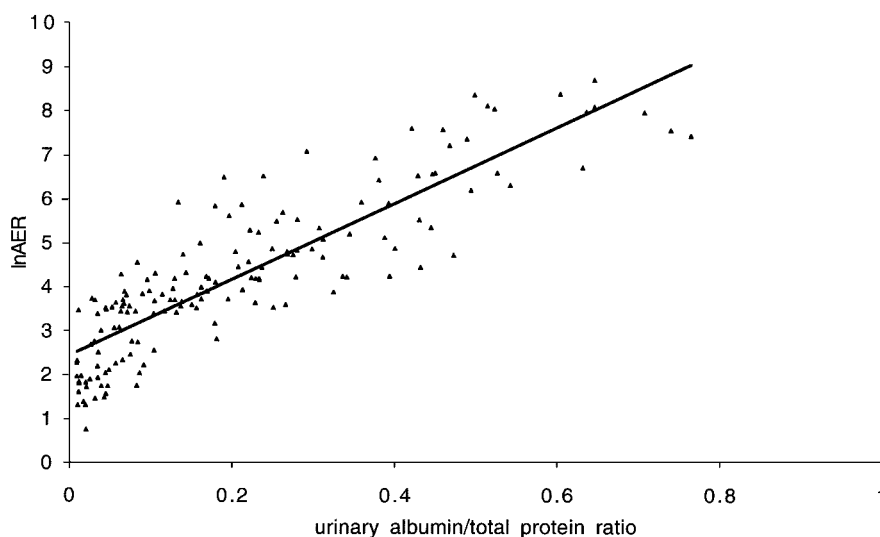


FIG. 2. Urinary albumin-to-total protein ratio was determined for optimal preparation of the samples. The ratio showed a strong correlation with lnAER.

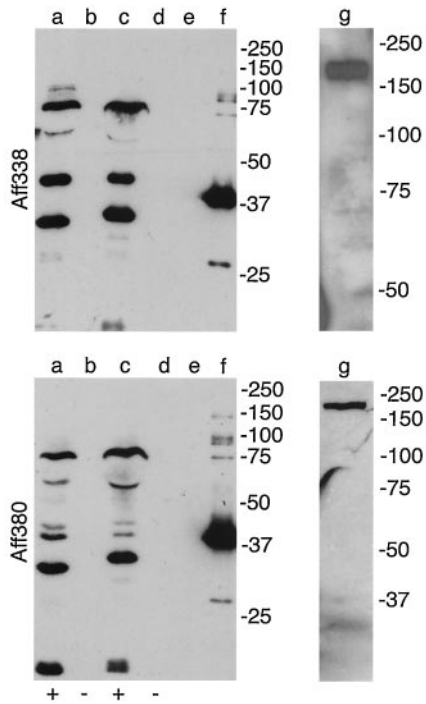


FIG. 3. Western blots showing the characteristic banding patterns of urine samples of individual nephrinuric diabetic patients (lanes *a* and *c*) stained with both anti-nephrin antibodies Aff338 and Aff380. The immunization antigen of the antibodies, α -435 (37 kDa), is in lane *f*, and full-length nephrin (185 kDa) is in lane *g*. Lanes *b* and *d* represent patient samples regarded as nonnephritic; lane *e* consists of Laemmli sample buffer without urinary sample. Molecular weights of the molecular standard are indicated on the right.

system, where degradation and reabsorption of proteins leaking from the glomerulus takes place (23,24). No protease inhibitors were added to the samples, so some nephrin degradation may also have occurred while the urine was collected and stored. Most of the nephritic urine demonstrated the characteristic banding pattern, but a few nephritic patients lacked some of the specific bands, which could mean that protease activities or tubular reabsorption may differ between individuals.

Our earlier results have shown that in diabetes induced experimentally by streptozotocin, nephrin was found in the urine earlier than albumin in the course of kidney damage (22). In human glomeruli, an alternatively spliced nephrin mRNA that lacks the transmembrane area exists, allowing production of a soluble form of the protein nephrin α (25). The observed fragments of nephrin in the present study may represent this form. Alternatively, the fragments could derive from the full-length nephrin from detaching podocytes. Nakamura et al. (26) found podocytes in the urine of micro- and macroalbuminuric type 2 diabetic patients, but patients with chronic renal failure failed to show urinary podocytes, suggesting that urinary podocytes may represent the active phase of diabetic nephropathy. In contrast, they did not find podocytes in the urine of normoalbuminuric patients (26). If the situation is the same in type 1 diabetes, then nephritic at the normoalbuminuric stage could represent secretion of nephrin α rather than detached podocytes. In support of this, our preliminary data show that fragments of other podocyte-specific proteins, including podocalyxin and podocin, are found only in a few nephritic urine sam-

ples of our study (unpublished data). It may be possible that before being shed, podocytes undergo a period of perturbed metabolism, leading to active secretion of its distinct molecular components.

Whether various human kidney diseases show changes in nephrin expression remains controversial (19,27–29). The expression is shown to be altered in effaced foot processes toward a more granular cytoplasmic localization, whereas in diseased glomeruli, the preserved foot processes show normal expression (20,30). Nephrin may be lost in urine and the granular staining could represent newly synthesized nephrin (30), or activation of podocyte foot process cytoskeleton could alter the surface distribution of nephrin (21). It is possible that primary reduction in nephrin may destabilize and disrupt the filtration slit structure and lead to podocyte loss, proteinuria, and nephritic.

Agents that modulate the renin-angiotensin system (RAS), such as ACE inhibitors or angiotensin-receptor antagonists, reduce proteinuria (31,32). In experimental models of diabetes, glomerular nephrin mRNA expression was reduced and the ACE inhibitors and angiotensin II antagonists were able to normalize the reduction in expression (17,33,34). In addition, ACE inhibitors and angiotensin II antagonists were able to normalize the ultrastructural changes in an experimental model of diabetes (35). In glomeruli of type 2 diabetic patients with nephropathy, Langham et al. (36) showed reduction of nephrin mRNA level, which was ameliorated by the use of an ACE inhibitor. These results propose that nephrin belongs to the as-yet-uncharacterized set of target genes for RAS modulation. In our study, we found no difference between the nephritic and nonnephritic patients in respect to use of antihypertensive agents. We expected the occurrence of nephritic in the Macro group to be higher compared with the other groups, but to our surprise, it was practically the same. It is interesting that 77% of the Macro patients were using RAS modifiers (ACE inhibitor or angiotensin II antagonist) and 18% other antihypertensive drugs, which may explain the results. The occurrence of nephritic in macroalbuminuric patients not using antihypertensive treatment, however, remains to be established.

Because nephrin is expressed by the pancreatic β -cells (16), one may speculate that the urinary immunoreactive nephrin represents nephrin spilled out from the serum and thus possibly excreted by the pancreas. Our preliminary results from Western blots of serum samples from five nephritic and three nonnephritic study patients did not show any immunoreactive protein bands, so the urinary nephrin most likely originates from the kidneys (data not shown).

What is the biological significance of detecting cell type-specific proteins and their degradation products in urine? In addition to adding the dynamic information on podocyte metabolism, structure, and function, these proteins may be used to profile different patient groups and to optimize pharmacologic intervention. They may also give hints to the currently open questions of the pathogenesis of diabetic nephropathy. In conclusion, one-third of the normoalbuminuric type 1 diabetic patients showed immunoreactive nephrin in the urine. The occurrence of nephritic was closely the same in the other groups tested.

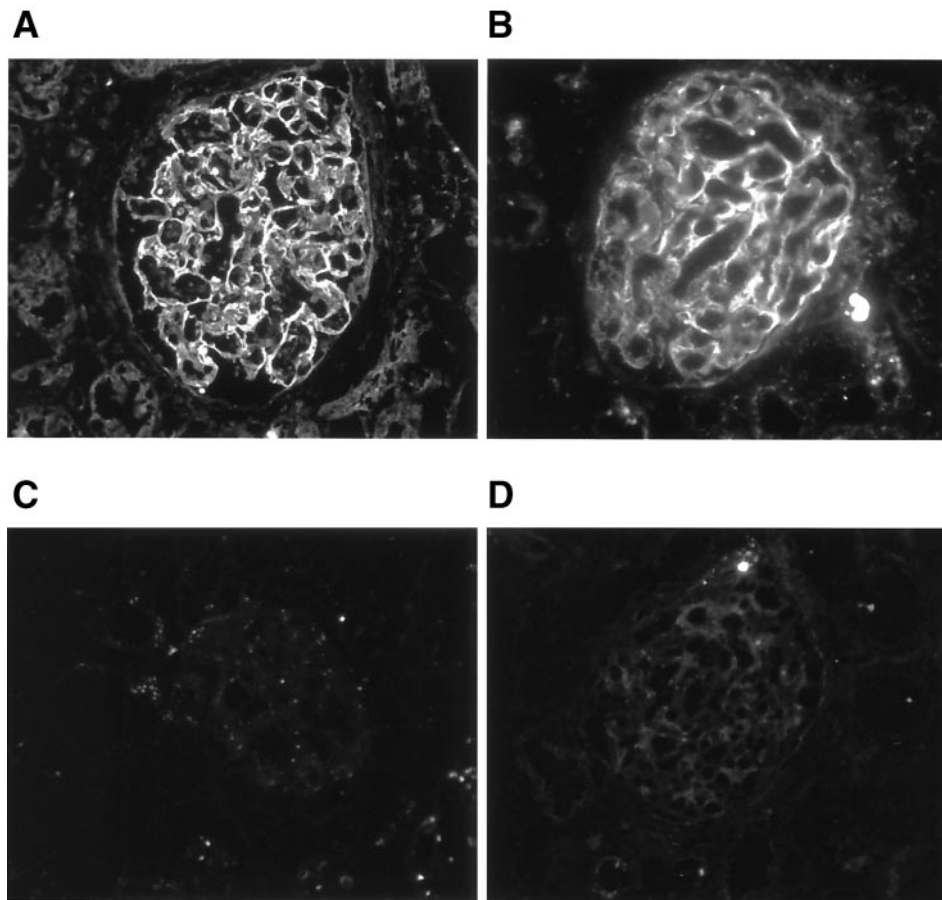


FIG. 4. Immunofluorescence stainings of human kidney cortex cryostat sections with anti-nephrin antibodies Aff338 (A) and Aff380 (B) showing a typical epithelial staining pattern. Control stainings without a primary antibody (C) or with an irrelevant anti-human glucagon antibody (D) exhibited negligible staining of the glomeruli. Magnification $\times 400$ in A and $\times 600$ in B.

Because all control subjects were nonnephritic, nephrinuria may be an early marker for damage of the glomeruli in type 1 diabetes.

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