Urinary and Kidney Podocalyxin and Podocin Levels in Diabetic Kidney Disease: A Kidney Biopsy Study

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Rationale & Objective: Diabetic kidney diseases (DKDs) are the most common cause of dialysis-dependent kidney disease around the world. Previous studies have suggested that urinary level of podocyte-associated molecules may predict the prognosis of DKD.

Study Design: Observational cohort.

Setting & Participants: 118 consecutive patients with biopsy-proven DKD; 13 nondiabetic patients with hypertensive nephrosclerosis as controls.

Predictors: Urinary podocalyxin and podocin levels were obtained by quantitative polymerase chain reaction and enzyme-linked immunosorbent assay (ELISA) and the corresponding intrarenal levels by western blotting.

Outcomes: Dialysis-free survival; kidney event-free survival; rate of kidney function decline in 12 months.

Analytical Approach: Correlation and time to event analysis.

Results: Urinary podocalyxin level was closely correlated with its messenger RNA (mRNA) level ($r = 0.562$, $P < 0.001$), but this did not predict the progression of DKD. Intrarenal podocalyxin level had only modest correlation with its urinary mRNA and ELISA levels, was an independent predictor of dialysis-free survival (adjusted HR, 1.85; 95% CI, 1.21-2.82; $P = 0.005$), and showed an insignificant trend of predicting kidney event-free survival (adjusted HR, 1.36; 95% CI, 0.94-1.95; $P = 0.10$). Urinary podocin level by ELISA had a modest correlation with the rate of kidney function decline ($r = 0.238$, $P = 0.01$) but did not predict dialysis-free survival.

Limitations: Small sample size; lack of serial measurement.

Conclusions: Intrarenal podocalyxin level, but not its urinary level, was an independent predictor of dialysis-free survival, whereas urinary podocin level by ELISA correlated with the rate of kidney function decline. Although intrarenal podocalyxin level has prognostic value, it may not be suitable for routine clinical use.

Visual Abstract included

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Diabetic kidney diseases (DKDs) are the most common cause of dialysis-dependent kidney disease around the world.[1] Recent studies have shown that 25%-40% of patients with type 1 diabetes and 5%-40% of patients with type 2 diabetes eventually develop DKD.[2] Although DKD is generally diagnosed by albuminuria and kidney function tests, novel biomarkers are needed for the prognosis, prediction of treatment response, and monitoring of DKD.[3,4]

Podocytes play a key role in the maintenance of normal kidney function and are the primary focus of many kidney diseases.[5,6] Various disease processes lead to podocyte damage and detachment from the glomerular basement membrane, and viable podocytes and their cellular fragments are detectable in the urine.[7,8] There is a wealth of literature showing that the urinary levels of many podocyte-associated molecules may serve as biomarkers for DKD and other kidney diseases.[9-11]

However, podocytes have many separate cellular compartments, and most of the previous studies focused on slit-diaphragm and foot-process proteins in the urine as biomarkers of kidney diseases.[12-16] For example, urinary levels of nephrin, a podocyte slit-diaphragm protein, correlated with the level of albuminuria and inversely with kidney function and is an earlier, more sensitive, and specific marker of diabetic nephropathy than microalbuminuria.[12,13] Urinary messenger (mRNA) level of podocin, another slit-diaphragm protein, helps evaluate podocyte loss and monitor treatment response of DKD.[14-16]

Podocalyxin is the major transmembrane protein specifically expressed on the apical side of podocyte (the so-called "top membrane") and is responsible for the regulation of podocyte morphology and glomerular permeability.[17,18] Preliminary studies showed that urinary podocalyxin level of DKD patients was higher than that of the control group, and the level positively correlated with glycemic control and urinary albumin excretion, indicating that urinary podocalyxin level might serve as a biomarker of DKD.[19,20] In the present study, we compared the role of urinary and intrarenal levels of podocalyxin and podocin (a prototype slit-diaphragm protein) biomarker in patients with biopsy-proven DKD.

METHODS

The study was approved by the Clinical Research Ethical Committee of the Chinese University of Hong Kong (approval number CRE-2019.283). All participants provided written inform consent for the study. All study procedures were in compliance with the Declaration of Helsinki.

PLAIN-LANGUAGE SUMMARY

Diabetic kidney diseases (DKDs) are the most common cause of dialysis-dependent kidney disease around the world. Previous studies suggested that the urinary level of podocyte-associated molecules may predict the prognosis of DKD. We studied 118 patients with biopsy-proven DKD. Their urinary and kidney levels of podocalyxin and podocin were measured. We found that podocalyxin level in the kidney, but not the urine, independently predicted dialysis-free survival. However, it is an invasive test and may not be suitable for routine clinical use.

Participants

We recruited 118 consecutive patients who had type 2 diabetes and kidney biopsy-proven diabetic nephropathy in our center. We also studied 13 nondiabetic patients with biopsy-proved hypertensive nephrosclerosis and 3 healthy kidney donors as controls. All kidney biopsy specimens were assessed by a single experienced pathologist and validated by an independent expert. Whole-stream early-morning timed (8 hours) urine was collected on the day of kidney biopsy. We also reviewed the participants' demographic and clinical data including serum creatinine and proteinuria. The estimated glomerular filtration rate (eGFR) was calculated by the Chronic Kidney Disease Epidemiology Collaboration equation.[21]

RNA Extraction

The method of mRNA extraction and quantification in urinary sediment has been described previously.[13] Briefly, urine samples were centrifuged immediately after collection at 4°C for 15 min, 3,200*g*. The supernatant was removed, the pellet suspended in 1.5 mL diethyl pyrocarbonate-treated phosphate-buffered saline, and then centrifuged at 12,000*g* for 5 min at 4°C. The washed pellet was resuspended in lysis buffer (RNeasy; Qiagen), and then kept frozen at −80°C until RNA extraction. The urinary pellet was then purified using an RNeasy mini kit (Qiagen), and cDNA was prepared with the SuperScript IV First-Strand Synthesis System (ThermoFisher).

RNA Preparation and Real-Time Quantitative Polymerase Chain Reaction (PCR)

Quantitation of podocin mRNA was performed with the StepOnePlus real-time PCR system (Applied Biosystems) using TaqMan Fast Advanced Master Mix (ThermoFisher) in a final volume of 10 μL per reaction. Commercially available TaqMan primers and probes, including 2 unlabeled PCR primers and one fluorescein amidite dye-labeled TaqMan minor groove binder probe, were used for both target genes (all from ThermoFisher). Each sample was run in triplicate. Results were analyzed with the use of Sequence Detection software, version 1.9 (Applied Biosystems). Gene expression for each signal was calculated by using the difference-in-threshold-cycle procedure. For the quantification of the target mRNA abundance, differences of threshold cycles between target genes were calculated. The cDNA standard curves generated from known concentrations of synthetic DNA oligonucleotides (all from ThermoFisher) that were identical in sequence to the corresponding target were constructed using these serially diluted standards. Assays were accepted only if R^2 was 0.97 for the standard curve. cDNAs of known sequence and concentration were used as standards for each assay. Quantitation of podocalyxin mRNA was performed using the Quantstudio 3D Digital PCR system (Applied Biosystems) including the chip loader, ProFlex thermal cycler, and chip reader. Reactions were prepared in 15 μL volumes with Quantstudio 3D Digital PCR Mastermix v2 (Applied Biosystems), TaqMan gene expression assays (podocalyxin: Hs00210532_m1). The concentration of podocalyxin was calculated using Quantasoft software (Applied Biosystems).

Urine Podocin and Podocalyxin Levels

Urinary supernatant podocin and podocalyxin levels were detected by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Bioassay Technology Laboratory) following the manufacturer's protocol. The enzymatic reaction was detected at 450 nm in an automatic microplate reader (Spectrafluorplus; Tecan) and adjusted to urinary creatinine level as measured by a creatinine colorimetric assay kit (MilliporeSigma). Each sample was measured in duplicate, and all measured creatinine concentrations were within range of the standard curve.

Intrarenal Podocin and Podocalyxin Levels

Intrarenal podocin and podocalyxin levels were determined by western blotting with β-actin level used as the reference. The protein electrophoresis, transfer apparatus, and acrylamide gel were obtained from Bio-Rad. The polyvinylidene fluoride membrane, protein detection reagent, and radiograph films were obtained from Bio-Rad. Total protein from the kidney biopsy specimen was lysed with radioimmunoprecipitation assay buffer containing protease inhibitors. The protein concentrations were quantified with a BCA Protein Assay Kit (Beyotime Institute of Biotechnology). Individual proteins were then separated from 20 μg of total protein extract by acrylamide gel electrophoresis in Mini-PROTEAN Cell and transferred to Hybond-P polyvinylidene fluoride membrane. The membranes were then probed with primary antibodies against podocin (1:1,000, Abcam), podocalyxin (1:1,000, Abcam), and β-actin (1:1,000, Abcam). The corresponding secondary antibody was obtained from Abcam. The membrane was exposed to Amersham Hyperfilm Blue. The areas of the bands were estimated with the Image J software. The protein expression level of a sample was calculated by dividing the area of the protein of interest by the area of β-actin of the same sample.

Table 1. Baseline Demographic and Clinical Data

	DKD	HTN	CTL	*P*
No. of patients	118	13	3	
Sex (M:F)	80:38	6:7	3:0	< 0.001[a]
Age (y)	61.3 ± 12.2	61.3 ± 13.8	56.6±0.9	0.85[b]
Serum creatinine (μmol/L)	217.9 ± 162.2	282.3 ± 162.6	63.0±15.6	0.14[b]
eGFR (mL/min/1.73 m^2)	41.4 ± 31.3	28.1 ± 21.8	110.3 ± 15.8	< 0.001[b]
Proteinuria (g/d)	2.5 (1.7-4.5)	0.6 (0.4-2.4)	—	0.01[c]
Histological damage (%)				
glomerulosclerosis	32.7 ± 21.7	25.5 ± 19.3	—	0.04[b]
tubulointerstitial fibrosis	30.2 ± 17.4	32.5 ± 20.4	—	0.58[b]

Abbreviations: CTL, healthy control group; DKD, diabetic kidney disease; eGFR, estimated glomerular filtration rate; F, female; HTN, hypertensive nephrosclerosis; M, male.
Data are presented as mean ± standard deviation or median (interquartile range) and compared between the DKD and HTN groups by.
[a]χ^2 test
[b]*t* test
[c]Mann-Whitney *U* test.

Morphometric Study of Kidney Biopsy

The method of morphometry study of kidney scarring has been described in previous studies.[22,23] Briefly, Jones' silver staining was performed on 5 μm thick sections of kidney biopsy specimen. Semiquantitative computerized image analysis was performed with the Leica Twin Pro image analysis system (Leica Microsystems), which was connected to a Leica DC500 digital camera on a Leica DMRXA2 microscope with a ×40 objective (final calibration: 0.258 mm/pixel). Image analysis was performed with MetaMorph 4.0 image-analyzing software (Universal Imaging Corporation). Ten glomeruli and 10 randomly selected areas were assessed in each patient's sample and the average percentage of scarred glomerular and tubulointerstitial areas, as represented by the area with positive silver staining, were computed.

Outcome Measures

All patients were followed for at least 12 months. All patients were followed by nephrologists and their treatment was not affected by the study and was only adjusted according to clinical need of the individual patients. Kidney function was monitored every 3 months. The primary outcome measures were dialysis-free survival and kidney event-free survival. Kidney event was defined as death of any cause, need for dialysis, or ≥40% decline in eGFR as compared to baseline. Secondary outcome measure includes the rate of eGFR decline, which was calculated by the least-square regression method.

Statistical Analysis

Statistical analysis was performed using SPSS for Windows software version 17.0. All the results are presented in mean ± standard deviation for normally distributed data and median (lower and upper quartiles) for the others. Because the data of gene expression levels were highly skewed, we used the Mann-Whitney U test to compare gene expression levels between groups and Spearman's rank-order correlations to test associations between gene expression levels and other parameters. Data were further analyzed with univariate and multivariable Cox regression analysis for dialysis-free survival and kidney event-free survival. In addition to podocin and podocalyxin level quartiles, the multivariable Cox regression model was constructed by age, baseline eGFR, proteinuria, severity of glomerulosclerosis, and tubulointerstitial fibrosis. A *P* value of below 0.05 was considered statistically significant. All probabilities were 2-tailed.

RESULTS

A total of 118 patients with biopsy-proven DKD were recruited. We also studied 13 nondiabetic patients with biopsy-proven hypertensive nephrosclerosis and 3 healthy kidney donors as controls. Their baseline demographic and clinical characteristics are summarized and compared in Table 1. For the DKD group, the average duration of diabetes was 6.0 ± 2.0 years. Their average hemoglobin A_{1c} was 6.7 ± 0.8%; 47 patients (39.8%) were receiving insulin therapy.

Urinary and Intrarenal Podocyte Marker Levels

The urinary and intrarenal levels of podocin and podocalyxin are summarized in Table 2. Representative western blot images are shown in Fig 1. Urinary and intrarenal levels of podocin and podocalyxin were further compared according to baseline chronic kidney disease stage, proteinuria level, and severity of glomerulosclerosis (Fig 2). In essence, urinary podocalyxin mRNA level, but not its urinary level measured by ELISA or intrarenal level, correlated with the severity of proteinuria. Urinary podocalyxin mRNA level was also marginally higher in patients with 25%-50% glomerulosclerosis than those with >25% or >50% glomerulosclerosis. The internal correlations between urinary and intrarenal podocyte marker levels in the DKD group are summarized in Table 3.

Relationship With Clinical and Histological Parameters

The relationship between podocyte marker levels and clinical and pathological parameters of the DKD group are summarized in Tables 4 and 5. In essence, there were

Table 2. Urinary and Intrarenal Podocyte Marker Levels

	DKD	HTN	CTL	*P*
Podocin				
Urinary level by ELISA (ng per mmol-Cr)	47.12 (11.05-178.64)	5.83 (1.90-19.48)	36.46 (21.44-52.50)	0.005
Urinary mRNA level (copy number/μL)[a]	911.48 (225.9-3125.25)	110.17 (51.97-339.94)	436.60 (301.05-622.28)	0.71
Intrarenal level[a]	39,603.5 (37,049.0-45,179.3)	41418.5 (37,957.5-43,740.5)	—	0.82
Podocalyxin				
Urinary level by ELISA (ng per mmol-Cr)	1.44 (1.23-1.93)	1.38 (1.05-2.10)	1.24 (1.19-2.93)	0.12
Urinary mRNA level (copy number/μL)[a]	251.15 (57.79-697.31)	90.42 (30.91-506.05)	46.89 (35.44-73.06)	0.49
Intrarenal level[b]	24,813.0 (19,823.0-29,810.0)	20953.5 (19,231.0-22,153.0)	—	0.52

Abbreviations: Cr, creatinine; CTL, healthy control group; DKD, diabetic kidney disease; ELISA, enzyme-linked immunosorbent assay; HTN, hypertensive nephrosclerosis; mRNA, messenger RNA.
Data are presented as median (interquartile range) and compared between the DKD and HTN groups by Mann-Whitney *U* test.
[a]copy number per μL of resuspended sediment
[b]relative band density in western blotting.

modest but statistically significant correlations between the severity of tubulointerstitial fibrosis and urinary sediment mRNA levels of podocin and podocalyxin (but not the urinary levels measured by ELISA), whereas the amount of proteinuria had a modest correlation with urinary mRNA level of podocalyxin but not podocin. The severity of glomerulosclerosis had a modest correlation with intrarenal podocalyxin, but not podocin, level.

Relationship With Clinical Outcome

The DKD group was followed for an average of 19.0 ± 16.9 months. During this period, none of the patients died; 66 patients progressed to dialysis-dependent kidney failure, and another 21 patients had ≥40% decline in eGFR. The eGFR was measured 12.4 ± 5.8 times per patient, and the average rate of eGFR decline was -14.6 ± 19.9 mL/min/1.73 m^2 per year. Patients with low intrarenal podocalyxin level had significantly higher risk for developing the kidney end point as compared with those with high level (Figure 3). The relationship between urinary and intrarenal podocin and podocalyxin levels and clinical outcome by univariate analysis is summarized in Table 6. With univariate analysis, only intrarenal podocalyxin level, but neither its urinary level nor any podocin marker, was associated with dialysis-free survival and kidney event-free survival. In contrast, urinary podocin level measured by ELISA had a modest but significant correlation with the rate of eGFR decline. After adjusting the clinical parameters by multivariable Cox regression analysis, intrarenal podocalyxin level remained an independent predictor of dialysis-free survival (adjusted hazard ratio, 1.85; 95% confidence interval, 1.21-2.82; $P = 0.005$) (Table 7). In this model, baseline eGFR was the other independent predictor of dialysis-free survival. By a similar analysis, intrarenal podocalyxin level also had a trend of predicting kidney event-free survival (adjusted hazard ratio, 1.36; 95% confidence interval, 0.94-1.95; $P = 0.10$), but the result did not reach statistical significance (Table 7). By multiple linear regression model analysis, urinary podocin level was still independently correlated with the rate of eGFR decline (Table 7). In this series of analyses, proteinuria did not predict dialysis-free

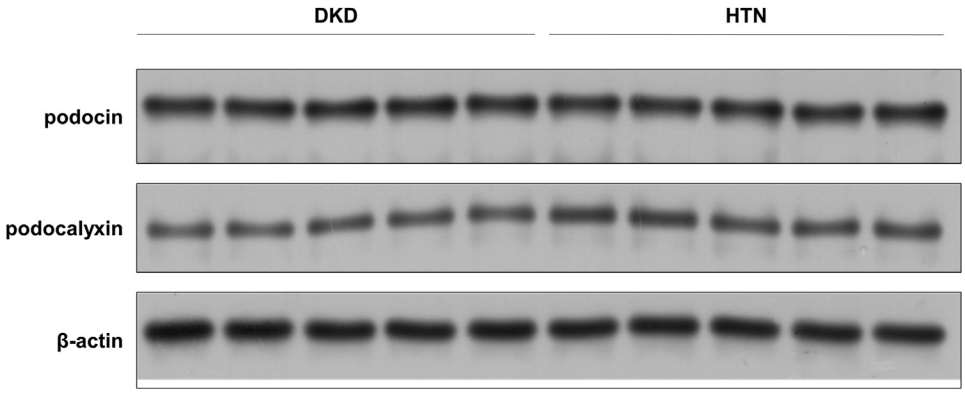

Figure 1. Representative images of western blots for intrarenal levels of podocin and podocalyxin. There was no significant difference in intrarenal podocin or podocalyxin levels between diabetic kidney disease (DKD) and hypertensive nephrosclerosis (HTN).

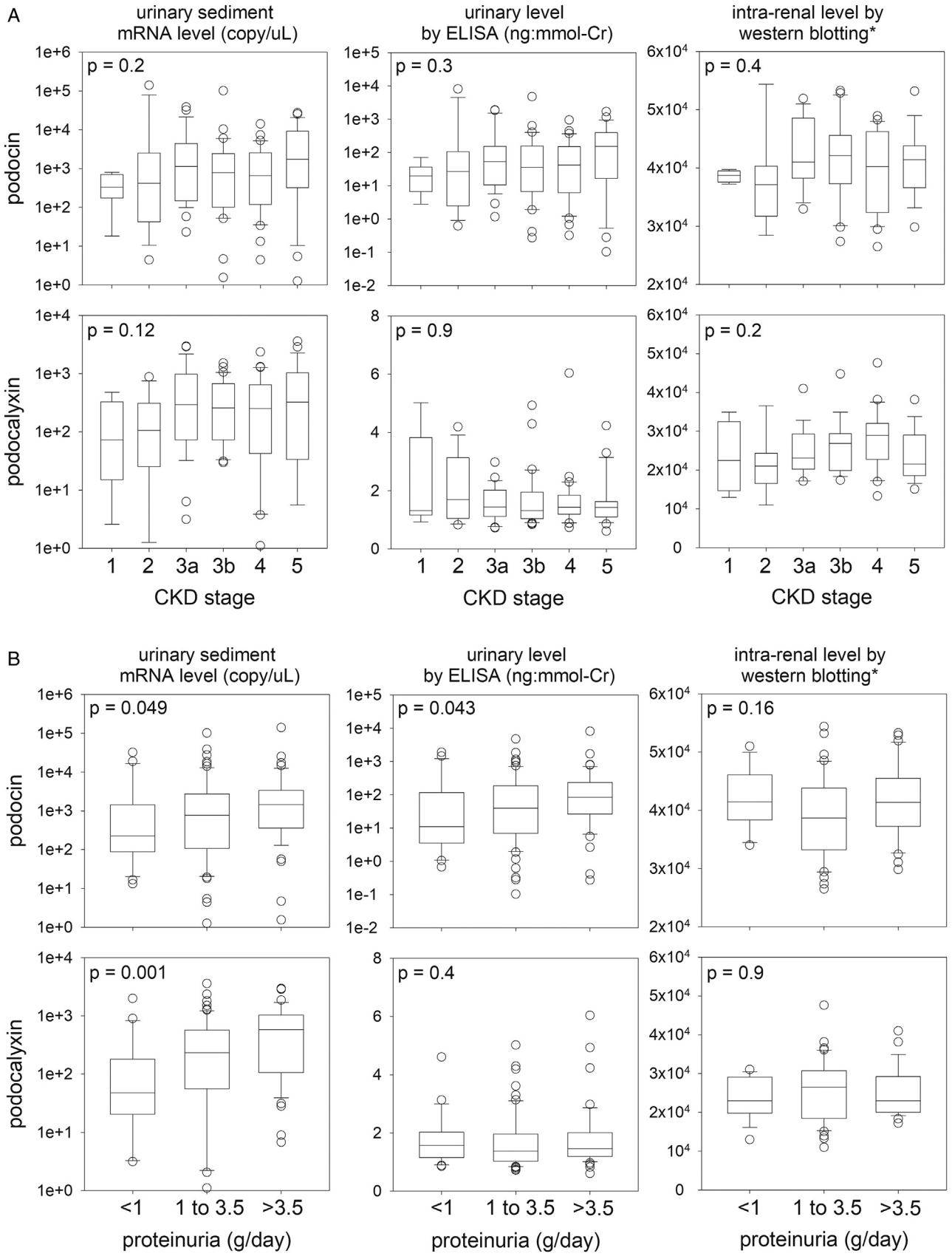

Figure 2. Urinary and intrarenal levels of podocin and podocalyxin grouped according to: (A) baseline chronic kidney disease (CKD) stage; (B) proteinuria level; and (C) severity of glomerulosclerosis. Data were compared by Kruskal-Wallis test. Abbreviations: Cr, creatinine; ELISA, enzyme-linked immunosorbent assay; mRNA, messenger RNA.*Arbitrary unit for the relative band density in western blotting.

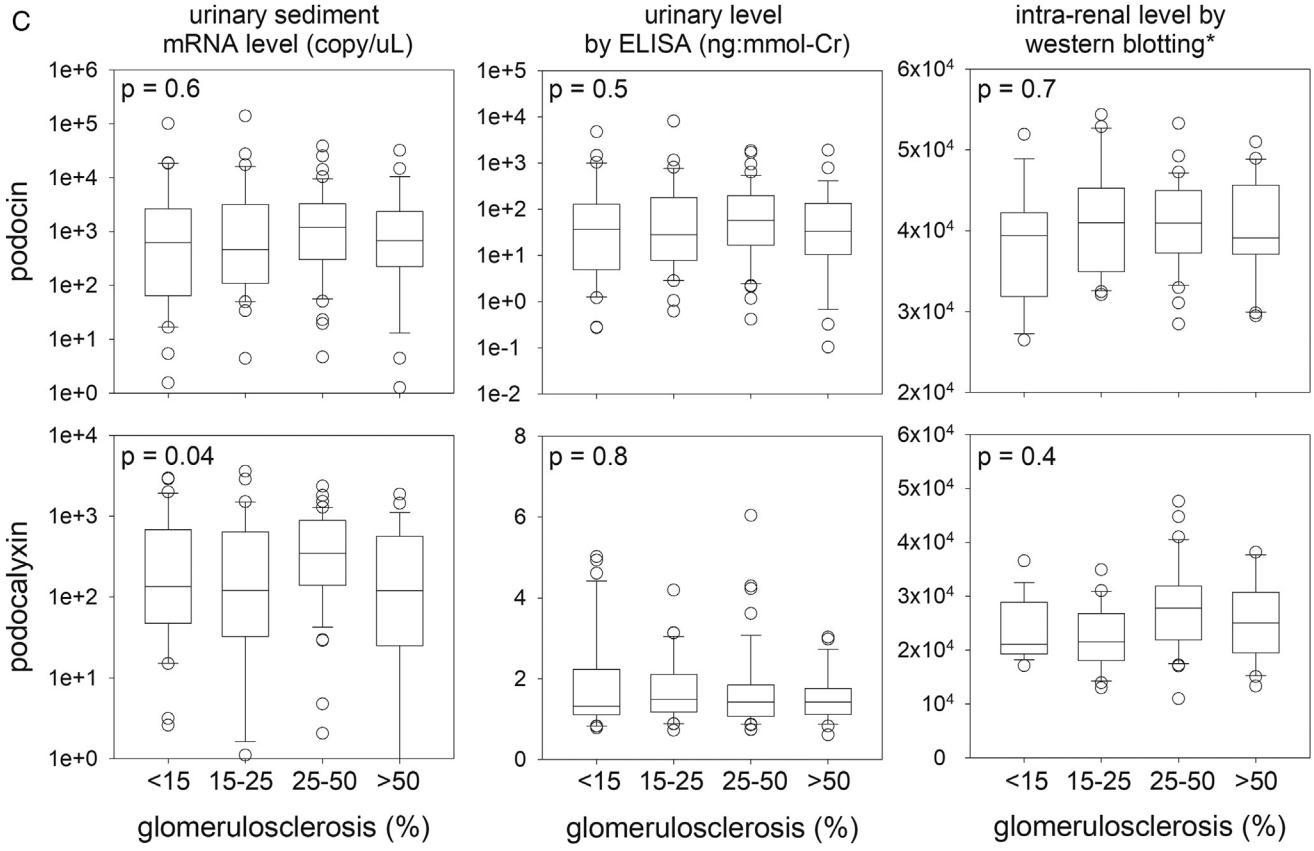

Figure 2. Continued.

survival, kidney event-free survival, or the rate of eGFR decline after adjusting for urinary and intrarenal podocin and podocalyxin level, baseline eGFR, and histological parameters.

DISCUSSION

In our study, we found that urinary podocalyxin level closely correlated with its mRNA level, but they did not appear to predict the progression of DKD. Intrarenal podocalyxin level, but not its urinary level, was an independent predictor of dialysis-free survival, whereas urinary podocin level determined by ELISA correlated with the rate of eGFR decline.

Our results are in line with but slightly different from that of previous studies, which showed that urinary podocin and podocalyxin are early-stage biomarkers of DKD. Similar to our present result, 2 previous studies reported that urinary podocin mRNA level was higher in patients with DKD than normal controls, and the level correlated with the severity of albuminuria and urinary podocyte count.[24,25] Urinary podocin mRNA-to-creatinine ratio had been considered as a marker of podocyte detachment and predicted the rate of subsequent kidney function decline.[14] As for podocalyxin, Kostavska et al[14] reported that urinary podocalyxin cutoff level of

Table 3. Internal Correlations Between Urinary and Intrarenal Podocyte Marker Levels in Diabetic Kidney Disease

	Urinary mRNA level	Intrarenal level
Podocin		
Urinary level by ELISA	r= 0.182, P = 0.04	r= -0.237, P = 0.03
Urinary mRNA level		r= -0.214, P = 0.04
Podocalyxin		
Urinary level by ELISA	r= 0.562, P < 0.001	r= -0.228, P = 0.04
Urinary mRNA level		r= -0.220, P = 0.04

Note: All data were compared by Spearman's rank correlation coefficient.
Abbreviations: ELISA, enzyme-linked immunosorbent assay; mRNA, messenger RNA.

Table 4. Relationship Between Podocin and Podocalyxin Levels

	Podocin and Podocalyxin
Urinary level by ELISA	r= 0.521, P < 0.001
Urinary mRNA level	r= -0.029, P = 0.74
Intrarenal level	r= 0.048, P = 0.65

Abbreviations: ELISA, enzyme-linked immunosorbent assay; mRNA, messenger RNA.

Table 5. Relationship Between Podocyte Marker Levels and Clinical and Pathological Parameters

	eGFR	Proteinuria	Glomerulosclerosis	Tubulointerstitial Fibrosis
Podocin				
Urinary level by ELISA	r= -0.056, P = 0.55	r= 0.176, P = 0.07	r= -0.090, P = 0.33	r= -0.081, P = 0.39
Urinary mRNA level	r= 0.022, P = 0.82	r= 0.073, P =0.42	r= -0.114, P = 0.22	r= 0.196, P = 0.02
Intrarenal level	r= -0.093, P = 0.41	r= 0.016, P = 0.89	r= 0.025, P = 0.82	r= -0.040, P = 0.72
Podocalyxin				
Urinary level by ELISA	r= -0.049, P = 0.60	r= -0.010, P = 0.92	r= -0.049, P = 0.60	r= -0.078, P = 0.41
Urinary mRNA level	r= -0.124, P = 0.18	r= 0.198, P = 0.04	r= -0.059, P = 0.53	r= 0.230, P = 0.01
Intrarenal level	r =-0.128, P = 0.25	r= -0.015, P = 0.90	r= 0.211, P = 0.05	r= 0.202, P = 0.07

Note: Data were compared by Spearman's rank correlation coefficient.
Abbreviations: eGFR, estimated glomerular filtration rate; ELISA, enzyme-linked immunosorbent assay; mRNA, messenger RNA.

43.8 ng/mL had 73.3% sensitivity and 93.3% specificity to detect early-stage DKD, and Hara et al[26] reported that urinary podocalyxin level was higher than the cutoff value in 53.8% of patients with diabetes at the normoalbuminuric stage. Similar to previous studies, we found that urinary podocalyxin level correlated with the degree of proteinuria in DKD.[15,19] However, although intrarenal podocalyxin level inversely correlated with the urinary podocalyxin mRNA and protein levels, only the intrarenal podocalyxin level correlated with the severity of glomerulosclerosis and dialysis-free survival. In contrast, urinary podocin level measured by ELISA, but not urinary podocin mRNA level as reported previously, correlated with the rate of eGFR decline.[20]

In this study, urinary podocin and podocalyxin mRNA levels had little correlation with the corresponding levels determined by ELISA. It is important to note that we measured podocin and podocalyxin levels in urinary supernatant by ELISA, whereas the mRNA levels were quantified from the cellular sediment. The 2 methods probably measure targets from different origins and may reflect different disease status. Specifically, mRNA in urinary sediment likely comes from denuded podocytes or their cellular fragments, whereas soluble podocin and podocalyxin in urinary supernatant probably originates from cellular leak following sublethal podocyte injury, but this hypothesis has not been proven.

Our results suggest that although podocin and podocalyxin are both podocyte-specific markers, they have different biological and clinical implications. From a biological point of view, podocalyxin is the major negatively charged protein synthesized by podocytes, and the expression is restricted to the apical membrane during the maturation of podocytes.[27] Podocin, on the other hand, is a

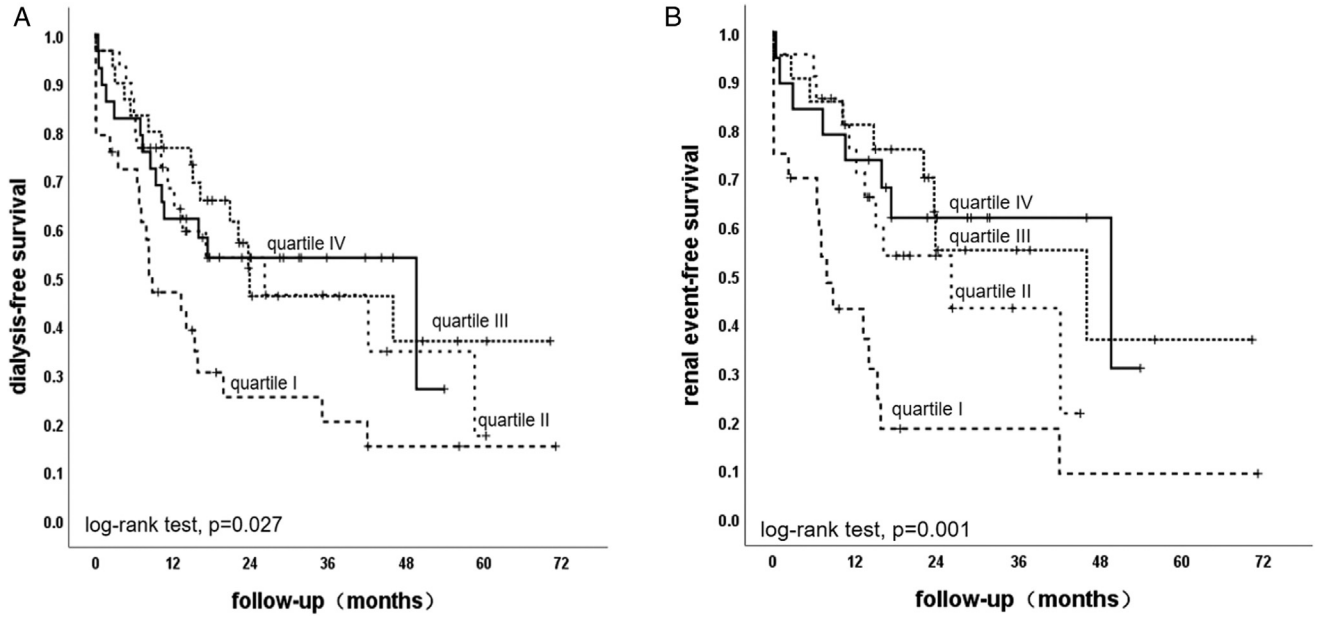

Figure 3. Kaplan-Meier plot of (A) dialysis-free survival; and (B) kidney event-free survival of the diabetic kidney diseases group. Patients were divided according to the quartiles of intrarenal podocalyxin level, with quartile I indicating the lowest level. Relative band intensity of podocalyxin western blotting for quartile I, II, III, and IV were <20,000, 20,000 to <25,000, 25,000 to <30,000, and ≥30,000, respectively. Data were compared with the log rank test.

Table 6. Relationship Between Podocyte Marker Levels and Clinical Outcome

	Dialysis-free Survival[a]	Kidney Event-free Survival[a]	Slope of eGFR Decline[b]
Podocin			
Urinary level by ELISA	0.97 (0.85-1.09), $P = 0.58$	0.95 (0.84-1.08), $P = 0.46$	$r = 0.238$, $P = 0.01$
Urinary mRNA level	1.24 (0.99-1.55), $P = 0.06$	1.17 (0.94-1.45), $P = 0.17$	$r = -0.093$, $P = 0.37$
Intrarenal level	1.25 (0.70-2.21), $P = 0.45$	0.86 (0.45-1.48), $P = 0.50$	$r = 0.002$, $P = 0.99$
Podocalyxin			
Urinary level by ELISA	2.76 (0.25-30.50), $P = 0.41$	6.84 (0.50-93.01), $P = 0.15$	$r = 0.026$, $P = 0.79$
Urinary mRNA level	1.32 (0.69-1.08), $P = 0.20$	0.92 (0.73-1.15), $P = 0.46$	$r = 0.193$, $P = 0.06$
Intrarenal level	1.81 (1.01-3.23), $P = 0.05$	2.01 (1.11-3.65), $P = 0.02$	$r = -0.170$, $P = 0.15$

[a]Unadjusted hazard ratio (95% confidence interval) by univariate Cox analysis.
[b]Spearman's rank correlation coefficient.

hairpin-like protein and is part of the slit-diaphragm protein complex responsible for connecting nephrin to the actin cytoskeleton under the cell membrane.[28] As to the clinical implication, intrarenal podocalyxin level significantly predicted dialysis-free survival and was less reliable in predicting kidney event-free survival, whereas urinary podocin level predicted the rate of eGFR decline. As an outcome measure, the recent consensus is that dialysis-free survival focuses on short-term progression and is biased toward rapid progressors, whereas the rate of eGFR decline put emphasis on the slow progressors in the long term.[29] Our result seems to indicate that intrarenal podocalyxin level is a marker of rapid progression, suggesting that this protein is induced in DKD, possibly as a result of podocyte stimulation or overactivation. However, the exact mechanism is unknown and deserves further study. In contrast, urinary podocin level represents the rate of progression for indolent cases. Although urinary podocalyxin level had a modest correlation with its intrarenal expression, it was not a predictor of dialysis-free survival, and our results would lead to the conclusion that urinary podocalyxin level may not be a suitable noninvasive biomarker for DKD.

Another interesting observation of our study was that intrarenal podocalyxin level inversely correlated with the urinary podocalyxin level, which is consistent with the notion that podocyte fragments are detached from diseased glomeruli into the urine. There is a wealth of literature to support that podocyte depletion and glomerulosclerosis have a direct relationship in the puromycin aminonucleoside-treated rat, and podocyte loss correlated with the progression of glomerular injury in type 2 diabetes.[30,31] Because the correlation between intrarenal and urinary podocalyxin levels was substantially higher than that of podocin, our result seems to suggest that cellular fragments, presumably from the apical membrane of diseased podocytes, rather than intact podocytes are the predominant component shed into the urine. In the present study, we did not measure the rate of intact podocyte loss in urine, and our hypothesis would need further studies to confirm. It is also important to note that the traditional method of detecting "intact" podocyte in urine uses cytospin techniques with immunofluorescence study by specific anti-podocalyxin antibody, which also detects podocyte apical membrane fragments and is prone to false-positive results.[32]

Table 7. Summary of Multivariable Analysis Models

	Multivariable Cox Regression Model for Dialysis-free Survival			Multivariable Cox Regression Model for Kidney Event-free Survival			Multiple Linear Regression Model for the Rate of eGFR Decline		
	AHR	95%CI	*P*	AHR	95% CI	*P*	Unstandardized B	95% CI	*P*
Age (1 y)	0.98	0.95-1.00	0.10	0.97	0.95-1.00	0.08	0.344	-0.069 to 0.757	0.10
Baseline eGFR (1 mL/min)	0.95	0.93-0.97	< 0.001	1.00	0.98-1.01	0.73	-0.196	-0.034 to -0.358	0.02
Proteinuria (1 g/d)	1.00	0.90-1.12	0.96	1.05	0.94-1.17	0.40	0.142	-1.565 to 1.849	0.87
Glomerulosclerosis (1%)	1.00	0.99-1.01	0.88	1.02	1.00-1.04	0.08	-0.112	-0.346 to 0.121	0.34
Tubulointerstitial fibrosis (1%)	1.03	1.01-1.04	0.01	1.00	0.98-1.03	0.97	-0.128	-0.465 to 0.209	0.45
Intrarenal podocalyxin (1000 unit[a])	1.85	1.21-2.82	<0.001	1.36	0.94-1.95	0.10			
Urinary podocin level (1 ng/mmol Cr)							1.846	0.166-3.527	0.03

Abbreviations: AHR, adjusted hazard ratio; CI, confidence interval; eGFR, estimated glomerular filtration rate.
[a]Relative band density in western blotting.

In addition, there are several other limitations of this study. First, this was a retrospective study on patients with DKD who underwent kidney biopsy. There may be referral (ie, patients with typical DKD might not be referred for biopsy) and selection bias (ie, patients with substantial kidney scarring and insufficient kidney tissue in the biopsy specimen would not be recruited). Second, this was a single-center study, and the sample size was small. The external validity of our findings is unknown, and the limited sample size precluded an extensive multivariable analysis. Similarly, additional analysis to determine the subgroup of patients for which podocyte markers provide valuable prognostic information was limited by the small sample size and number of events. In theory, urinary podocin and podocalyxin levels could also be measured by western blotting, but absolute quantification is less robust as compared to the simple ELISA assay. Unfortunately, the size of the available kidney biopsy tissue was small, and the ELISA test was not feasible. Because we only measured the urinary podocin and podocalyxin levels at 1 time point (ie, around the time of kidney biopsy), the intraindividual variability of the measurement is unknown, and it remains to be determined whether serial monitoring of urinary podocin or podocalyxin, either at mRNA or protein level, would provide additional prognostic information. Notably, introduction of effective treatment that reduces proteinuria or improves kidney function may lead to a change in the urinary podocyte marker levels, and further studies in this area are needed.

Nonetheless, our results show that the urinary podocin level may be a prognostic marker of DKD. Although the intrarenal podocalyxin level also has prognostic value, it does not appear to be a promising clinical marker in view of the invasive nature of the test, and urinary podocalyxin, either at mRNA or protein level, does not provide additional prognostic information. Our results also suggest that qualitative changes in various cellular compartments of the podocyte, rather than the change in the absolute podocyte number, may be an important pathological alteration in DKD.

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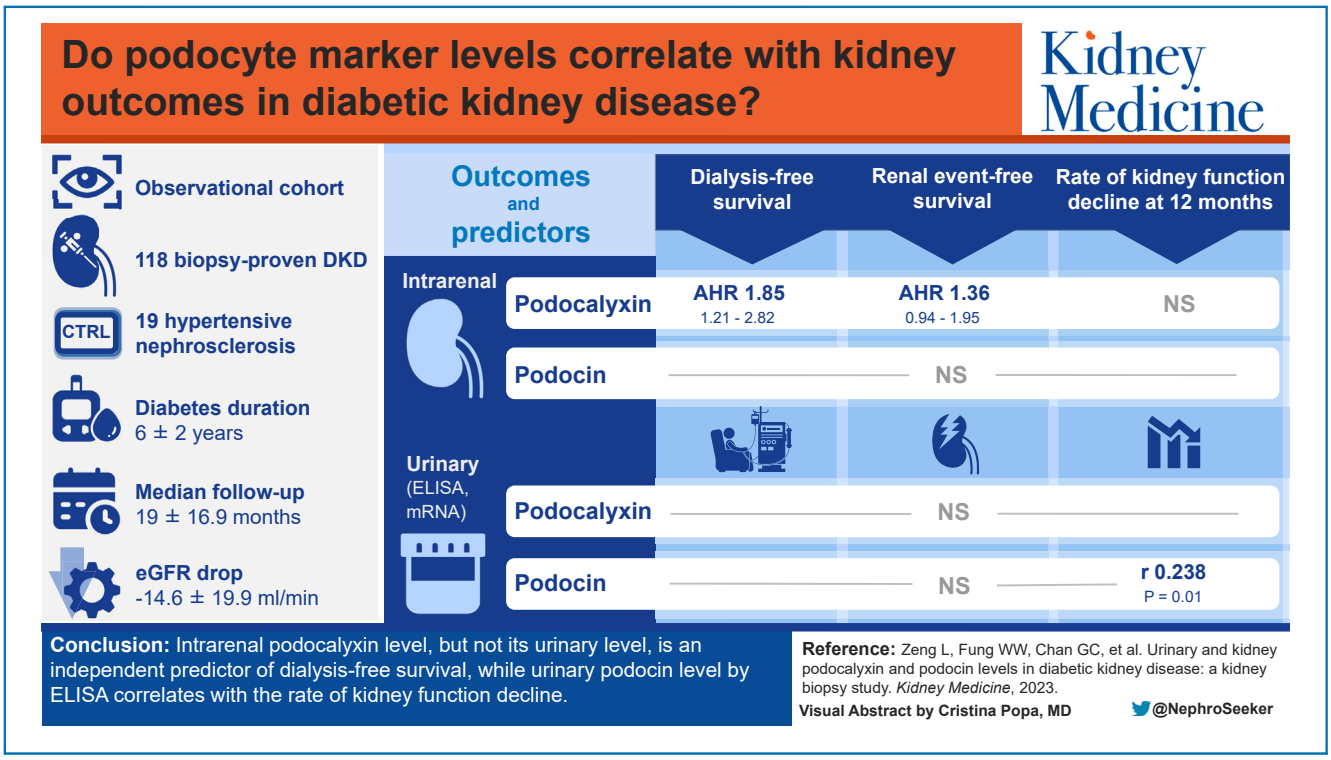
Do podocyte marker levels correlate with kidney outcomes in diabetic kidney disease?
Kidney Medicine
Observational cohort
118 biopsy-proven DKD
CTRL
19 hypertensive nephrosclerosis
Diabetes duration
6 ± 2 years
Median follow-up
19 ± 16.9 months
eGFR drop
-14.6 ± 19.9 ml/min
Outcomes and predictors
Dialysis-free survival
Renal event-free survival
Rate of kidney function decline at 12 months
Intrarenal
Podocalyxin
AHR 1.85
1.21 - 2.82
AHR 1.36
0.94 - 1.95
NS
Podocin
NS
Urinary
(ELISA, mRNA)
Podocalyxin
NS
Podocin
NS
r 0.238
P = 0.01
Conclusion: Intrarenal podocalyxin level, but not its urinary level, is an independent predictor of dialysis-free survival, while urinary podocin level by ELISA correlates with the rate of kidney function decline.
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