Excretion Patterns of Urinary Sediment and Supernatant Podocyte Biomarkers in Patients with Chronic Kidney Disease

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Akihiro Fukuda, Akihiro Minakawa, Yuji Sato, Hirotaka Shibata, Masanori Hara, and Shouichi Fujimoto

Key Points:
* Higher urinary sediment podocyte mRNA excretion is associated with proliferative-type glomerulonephritis indicating podocyte detachment.
* Higher urinary supernatant podocyte protein is associated with subepithelial dense deposit-type glomerulonephritis.
* These podocyte biomarkers have usefulness for the diagnosis and measurement of disease activity of glomerular diseases.

Abstract:
Background: Podocyte depletion causes glomerulosclerosis, and persistent podocyte loss drives progression to end-stage kidney disease. Urinary sediment podocyte (u-sed Pod) mRNA excretion and urinary supernatant podocyte (u-sup PCX) protein have been used to monitor disease activity in glomerular diseases. However, the differences in these markers among pathologies have not been investigated. We examined the roles of these markers in kidney diseases. Methods: From January 2013 to March 2016, early morning urine samples were collected from 12 healthy controls and 172 patients with kidney disease (minor glomerular abnormality with mild proteinuria and/or microscopic hematuria, n = 15; minimal change nephrotic syndrome [MCNS], n = 15; membranous nephropathy [MN], n = 15; IgA nephropathy [IgAN], n = 60; crescentic glomerulonephritis [Cres GN], n = 19; lupus nephritis [LN], n = 10; others, n = 38). We examined u-sed Pod mRNA excretion, u-sup PCX protein and the urinary protein:creatinine ratio (u-PCR). Results: U-sed Pod mRNA excretion was significantly correlated with u-sup PCX protein (r = 0.37, p < 0.001). Both u-sed Pod mRNA excretion and u-sup PCX protein were significantly correlated with u-PCR (r = 0.53, p < 0.001 and r = 0.35, p < 0.001, respectively). Interestingly, u-sed Pod mRNA excretion was significantly increased in proliferative-type glomerulonephritis-including IgAN with extracapillary proliferative lesions, Cres GN and LN class IV-and significantly correlated with the rate of crescent formation, whereas u-sup PCX protein was significantly increased only in MN and subepithelial dense deposit-type LN compared with controls. Conclusions: Higher u-sed Pod mRNA expression and u-sup PCX protein were associated with proliferative-type glomerulonephritis indicating podocyte detachment and subepithelial dense deposit-type glomerulonephritis, respectively. The results suggest that u-sed Pod mRNA excretion and u-sup PCX protein have usefulness for the diagnosis and measurement of disease activity with regard to glomerular diseases.

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Abstract

Background

Podocyte depletion causes glomerulosclerosis, and persistent podocyte loss drives progression to end-stage kidney disease. Urinary sediment podocyte (u-sed Pod) mRNA excretion and urinary supernatant podocyte (u-sup PCX) protein have been used to monitor disease activity in glomerular diseases. However, the differences in these markers among pathologies have not been investigated.

We examined the roles of these markers in kidney diseases.

Methods

From January 2013 to March 2016, early morning urine samples were collected from 12 healthy controls and 172 patients with kidney disease (minor glomerular abnormality with mild proteinuria and/or microscopic hematuria, n = 15; minimal change nephrotic syndrome [MCNS], n = 15;
membranous nephropathy [MN], n = 15; IgA nephropathy [IgAN], n = 60; crescentic glomerulonephritis [Cres GN], n = 19; lupus nephritis [LN], n = 10; others, n = 38). We examined u-sed Pod mRNA excretion, u-sup PCX protein and the urinary protein:creatinine ratio (u-PCR).

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U-sed Pod mRNA excretion was significantly correlated with u-sup PCX protein (r = 0.37, p < 0.001). Both u-sed Pod mRNA excretion and u-sup PCX protein were significantly correlated with u-PCR (r = 0.53, p < 0.001 and r = 0.35, p < 0.001, respectively). Interestingly, u-sed Pod mRNA excretion was significantly increased in proliferative-type glomerulonephritis—including IgAN with extracapillary proliferative lesions, Cres GN and LN class IV—and significantly correlated with the rate of crescent formation, whereas u-sup PCX protein was significantly increased only in MN and subepithelial dense deposit-type LN compared with controls.

Conclusions

Higher u-sed Pod mRNA excretion and u-sup PCX protein were associated with proliferative-type glomerulonephritis indicating podocyte detachment and subepithelial dense deposit-type glomerulonephritis, respectively. The results suggest that u-sed Pod mRNA excretion and u-sup PCX protein have usefulness for the diagnosis and measurement of disease activity with regard to glomerular diseases.
Introduction

Proteinuria and/or albuminuria have served as diagnostic and monitoring tools for kidney diseases in the clinic for many years. Proteinuria increases early in glomerular injury and can potentially serve as an early and predictive marker of future progression. However, proteinuria is caused by many mechanisms, including various forms of glomerular injury, tubule-interstitial injury, and physiologic processes that enhance glomerular filtration of protein (1). Proteinuria is, therefore, not a specific biomarker for any single kidney disease. However, it is a viable tool for monitoring kidney injury and the response to treatment.

Glomerular diseases, including diabetic kidney disease and hypertension, comprise more than 80% of the causes of end-stage kidney disease. Compelling data now support the concept that podocyte injury and depletion cause glomerulosclerosis, and that persistent podocyte loss drives most forms of
progression of glomerular diseases (2–18). Podocytes reside on the urinary space side of the glomerular basement membrane, so that as they detach or die, their products can be identified in urine. Thus, in the last quarter century, we and other investigators reported that podocyte products in urine (such as urinary sediment podocyte mRNA excretion and urinary supernatant podocyte protein) could be potential biomarkers of glomerular disease activity and progression (19–32). However, no study has investigated the differences in these urinary podocyte biomarkers. We examined the importance of these markers in various kidney diseases.

**Materials and Methods**

**Ethical considerations**

This study was conducted according to the principles of the Declaration of Helsinki and was approved by the Institutional Review Board of the University of Miyazaki Hospital (No. 2014-055). Informed consent was obtained from all subjects.

**Collection and histological evaluation of samples from patients with kidney disease**

From January 2013 to March 2016, urine samples were collected in the morning from 184 consecutive patients with kidney disease (minor glomerular abnormality with mild proteinuria and/or microscopic hematuria, n = 15; minimal change nephrotic syndrome [MCNS], n = 15; membranous nephropathy [MN], n = 15; IgA nephropathy [IgAN], n = 60; crescentic glomerulonephritis [Cres
GN], n = 19; lupus nephritis [LN], n = 10; others, n = 38 (mesangial proliferative glomerulonephritis [IgA negative], n = 7; IgA vasculitis, n = 5; focal segmental glomerulosclerosis, n = 4; secondary IgA nephropathy, n = 3; membranoproliferative glomerulonephritis, n = 3; interstitial nephritis, n = 3; nephrosclerosis, n = 2; AL amyloidosis, n = 2; post-infectious glomerulonephritis, n = 2; endocapillary proliferative glomerulonephritis, n = 1; light chain deposition disease, n = 1; C3 glomerulopathy, n = 1; obesity related glomerulopathy, n = 1; diabetic nephropathy, n = 1; bone marrow transplantation nephropathy, n = 1; familial nephropathy associated with hyperuricemia, n = 1) and 12 healthy volunteers. Minor glomerular abnormality cases were those with mild proteinuria and microscopic hematuria (mostly qualitative 1+, quantitative mean 0.3 g/gCr) found during physical examination, and those with minor glomerular changes as a result of renal biopsy. The healthy volunteers had never been diagnosed with any underlying disease in the past medical checkups and had not been diagnosed with any urinary abnormalities or renal dysfunction in the last year. Based on this information, we determined that the healthy volunteers did not have kidney disease or hypertension, and only urine samples were collected. The estimated glomerular filtration rate (eGFR) was estimated by the IDMS-MDRD method adjusted for the Japanese population (194 × serum creatinine\(^{-1.094}\) × age\(^{0.287}\) × 0.739 [if female]) (33). The clinical parameters of the patients with kidney disease and healthy controls are shown in Table 1. The urinary protein:creatinine ratio (u-PCR), urinary sediment podocyte (podocin) mRNA factored urinary creatinine concentration (u-sed Pod mRNA), and urinary supernatant podocalyxin protein factored urinary creatinine concentration.
(u-sup PCX protein) were measured. The Oxford classification (34) system was used to evaluate the histological findings of patients with IgAN. The minimal number of glomerular profiles evaluated per section was eight, according to the Oxford classification system. The rate of crescent formation in patients with Cres GN was counted as the percentage of glomeruli with cellular and fibrocellular crescents. MN was classified into stages I–IV according to Churg’s stage classification (35) based on electron microscopic findings. LNs were evaluated for the presence of subepithelial and subendothelial deposits based on electron microscopic findings. Histology slides were evaluated by two investigators blinded to sample identity.

**Extraction of RNA from human urinary sediment**

Urine samples were collected in the morning and centrifuged at 4°C for 15 min at 3200 × g in a tabletop centrifuge. The supernatant was removed, the pellet suspended in 1.5 mL of diethyl pyrocarbonate-treated phosphate-buffered saline, and centrifuged at 12000 × g for 5 min at 4°C. The washed pellet was resuspended in RLT/β-mercaptoethanol buffer (RNeasy Kit; Qiagen, Germantown, MD) and frozen at −80°C until RNA extraction (16, 23, 28).

**RNA preparation and reverse transcription-quantitative polymerase chain reaction**

Total urinary sediment was purified using an RNeasy Mini Kit (cat. No. 74106; Qiagen). cDNA was transcribed from sample total RNA using a High-Capacity cDNA Reverse Transcription Kit
Quantitation of the podocin mRNA abundance was performed with a LightCycler 96 system (Roche Molecular Systems, Inc., Mannheim, Germany) using FastStart Essential DNA Probe Master Mix (Roche Molecular Systems, Inc.) in a final volume of 10 μL per reaction. The TaqMan probe (Applied Biosystems) used was for human NPHS2 (podocin; cat. no. Hs00922492_m1). Data were from 2 μg samples of cDNA measured in duplicate. cDNA standard curves were constructed using these serially diluted standards as described previously (16, 23, 28). The concentration of urinary sediment podocin mRNA was standardized by the creatinine concentration and was expressed as M/g creatinine.

**Quantitation of urinary supernatant podocalyxin protein concentration**

U-sup PCX was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) as described previously (31, 36). To construct the sandwich-type ELISA, the protein-G-bound fraction from ascitic fluid was used as the capture antibody for ELISA plates and was labelled with horseradish peroxidase (HRP). The urine samples were mixed and incubated with an equal volume of sample buffer (0.4 M N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid–sodium hydroxide buffer containing 0.04 M ethylenediaminetetraacetic acid and 0.4% Triton X-100, pH 7.0). ELISA was performed using 100 μL of treated urine samples. The u-sup PCX concentration was standardized to the creatinine concentration and expressed as ng/μmol creatinine.
**Statistical analysis**

Statistical analysis was performed using Prism software, version 6.0 (GraphPad Software, Inc., La Jolla, CA). The clinical parameters of the patients with kidney disease and urinary measurements are given as means ± standard deviation. Differences between the two groups were evaluated using the Mann–Whitney U test, and those among more than two groups were evaluated by Kruskal–Wallis test. When the result of the Kruskal–Wallis test was significant, the Dunn test was performed for post hoc analysis. Correlations between parameters were assessed by single regression analysis (Spearman rank correlation). A p-value < 0.05 was considered indicative of statistical significance.

**Results**

**Correlation between urinary sediment podocyte (podocin) (u-sed Pod) mRNA excretion, urinary supernatant podocyte (podocalyxin) (u-sup PCX) protein, and proteinuria**

Table 1 shows the clinical parameters of the healthy controls (n = 12) and kidney disease patients (minor glomerular abnormality with mild proteinuria and/or microscopic hematuria, n = 15; MCNS, n = 15; MN, n = 15; IgAN, n = 60; Cres GN, n = 19; LN, n = 10; and others [other glomerular diseases and interstitial nephritis: details are provided in the Methods], n = 38) in this cross-sectional study. A low serum albumin level and nephrotic range of proteinuria were observed in MCNS and MN, and the eGFR was decreased in Cres GN. The anti-hypertensive and immunosuppressive medications used at the time of urine collection are shown in Table 1.
U-sed Pod mRNA excretion was significantly correlated with u-sup PCX protein \( (r = 0.37, p < 0.001) \). U-sed Pod mRNA excretion and u-sup PCX protein were significantly correlated with u-PCR \( (r = 0.53, p < 0.001 \text{ and } r = 0.35, p < 0.001, \text{ respectively}) \) (Figure 1). These data suggest that urinary podocyte biomarkers are correlated with proteinuria in kidney diseases.

**U-sed Pod mRNA excretion and u-sup PCX protein in glomerular diseases**

We selected representative glomerular diseases (134 patients; minor glomerular abnormality with mild proteinuria and/or microscopic hematuria, \( n = 15 \); MCNS, \( n = 15 \); MN, \( n = 15 \); IgAN, \( n = 60 \); Cres GN, \( n = 19 \); LN, \( n = 10 \)) to assess the difference between u-sed Pod mRNA excretion and u-sup PCX protein. Diseases with fewer than eight cases were excluded due to the difficulty of statistical analysis. Proteinuria was significantly increased in all of the glomerular diseases. U-sed Pod mRNA excretion was also significantly increased in all of the glomerular diseases compared with the controls, and was further increased in IgAN (29-fold), Cres GN (76-fold), and LN (190-fold), whereas u-sup PCX protein was significantly increased only in MN (5.1-fold) and LN (5.5-fold) compared with the controls (Figure 2). These data indicate that u-sed Pod mRNA excretion and u-sup PCX protein could provide different information.

**U-sed Pod mRNA excretion, u-sup PCX protein, and relationship to histopathological findings in glomerular diseases**
First, we evaluated the proliferative glomerular diseases, IgAN and Cres GN. U-sed Pod mRNA excretion was significantly increased only with extracapillary proliferative lesions in the Oxford classification, whereas u-sup PCX protein did not differ significantly in any lesions in IgAN (Figure 3A). Furthermore, u-sed Pod mRNA excretion, but not u-sup PCX protein, was correlated significantly with the rate of crescent formation (n = 17, r = 0.56, p = 0.02, and n = 17, r = -0.12, p = 0.65, respectively; two cases were excluded because renal biopsy was not performed) in Cres GN (Figure 3B and 3C). These results suggest that u-sed Pod mRNA excretion is strongly associated with extracapillary proliferative lesions, as we have reported previously (23, 27).

Next, we evaluated lupus nephritis, which has proliferative and non-proliferative forms. U-sed Pod mRNA excretion and u-sup PCX protein were significantly increased in patients with LN compared with the controls (190 and 5.5-fold, respectively). We examined differences in urinary podocyte markers among pathological classifications of LN. Table 2 shows the clinical parameters, urinary biomarkers, and areas of dense deposition in 10 patients with LN. The ratio of males to females was 1:9. The histological classification was class III in four cases, IV in three cases, and V in three cases. The mean eGFR was >60 mL/min/1.73m²; there was nephrotic-range proteinuria in class IV. U-sed Pod mRNA excretion was increased in class IV, whereas u-sup PCX protein was increased in the presence of subepithelial dense deposition but not subendothelial dense deposition (Figure 4A, 4B and Table 2). Although the sample size was small, these data suggest that higher u-sed Pod mRNA excretion and u-sup PCX protein might be associated with proliferative-type GN (class III+IV: 260-
fold vs control) and subepithelial dense deposit GN (6.9-fold vs control), respectively. We found that u-sup PCX protein was increased in subepithelial dense deposit-type LN, we next validated this finding in MN. Table 3 shows the clinical parameters, urinary biomarkers, and histological stages of 15 patients with MN. The ratio of males to females was 6:9. The histological stage was class I in four cases, II in five cases, III in six cases, and IV in no case. The mean eGFR was 67.2 mL/min/1.73m² and mean proteinuria was 3.4 g/gCr. Both u-sed Pod mRNA excretion and u-sup PCX protein were increased compared with the controls. There was no difference in the two podocyte markers according to classification stage. (Figure 4C, 4D and Table 3), but u-sup PCX protein in MN was increased 5.1-fold, compared to the controls and to proliferative glomerular disease (1.6-fold for IgAN and 1.4-fold for Cres GN). Furthermore, when IgAN, Cres GN, MN, and LN cases were divided into mesangial proliferative type, extracapillary proliferative type, and subepithelial dense deposit type (LN class IV was excluded because it corresponded to both the proliferative and non-proliferative types), u-sed pod mRNA excretion was increased in the extracapillary proliferative type, and u-sup PCX protein was increased in the subepithelial dense deposit type (Figure 5). These results are consistent with the LN, in which higher u-sed Pod mRNA excretion and u-sup PCX protein were associated with proliferative-type GN and subepithelial dense deposit-type GN, respectively.

There was a significant correlation between urinary podocyte markers (u-sed Pod mRNA excretion and u-sup PCX protein) and proteinuria (Figure 1). Therefore, we evaluated the association
between podocyte depletion and proteinuria in MCNS, which had the highest frequency of proteinuria. Supplemental Table 1 shows the clinical parameters, urinary biomarkers, and the presence or absence of relapse in 15 patients with MCNS. The ratio of males to females was 7:8. The mean eGFR was 51.7 mL/min/1.73m² and mean proteinuria was 6.7 g/gCr. Four of the fifteen patients did not have nephrotic–range proteinuria, but these patients had been on steroids or other immunosuppressive drugs at the time of urine sample collection. No patient was found to have focal segmental glomerulosclerosis during the observation period, and urinary podocyte markers did not differ between patients with and without relapse. U-sed Pod mRNA excretion was significantly correlated with proteinuria, whereas u-sup PCX protein was not (Supplemental Figure 1). These results suggest that patients with massive proteinuria may have some podocyte detachment even in MCNS, although this may be influenced by the fact that many patients have poor renal function (mean e-GFR < 60ml/min/1.73m²) at the time of urine collection.

Discussion

We assessed u-sed Pod mRNA excretion and u-sup PCX protein in various kidney diseases. Both of these biomarkers have potential for the diagnosis and monitoring of glomerular diseases (19–32). We reported detection of specific biomarkers in urine pellets using mRNA technology (11–18, 21–28). This approach has several advantages in that it is potentially quantitative, is sensitive and specific, and can be multiplexed to measure several mRNAs simultaneously. We also reported detection of
urinary podocyte mRNA in rat models of several glomerular diseases and human diseases, including IgAN, anti-neutrophil cytoplasmic antibodies-associated glomerulonephritis, and diabetic nephropathy. Furthermore, the glomerular podocyte loss rate was quantitatively related to the podocyte detachment rate measured by urinary pellet mRNAs (11–18, 21–28). These reports suggested that urinary sediment podocyte mRNA is a potential biomarker for the monitoring of glomerular diseases ranging from mild to severe podocyte detachment. Hara et al. (29) reported two structural elements in urine sediment—podocalyxin-positive cells and podocalyxin-positive subcellular granular structures—using an anti-podocalyxin antibody. These granular structures originated from podocyte microvilli or vesicle-like structures derived from podocytes according to light microscopy and electron microscopy. These granular structures in urine were derived from the apical portion of podocyte cell membranes and quantified by ELISA (u-PCX). They concluded that the detection of microvesicles in urine by this ELISA (u-PCX) could be useful for diagnosing and monitoring early-stage glomerular disease (29–32).

U-sed Pod mRNA excretion was significantly increased in all glomerular diseases, whereas, u-sup PCX protein was increased only in MN and LN compared with the controls. IgAN with extracapillary proliferative lesions, Cres GN and LN class IV, in which we expected severe podocyte detachment, showed significantly increased u-sed Pod mRNA excretion. In addition, u-sed Pod mRNA excretion, but not u-sup PCX protein, was correlated significantly with the rate of crescent formation in Cres GN. These results suggest that u-sed Pod mRNA excretion correlates with
histological podocyte depletion and may be useful as a marker of podocyte detachment. Furthermore, u-sed Pod mRNA excretion was significantly correlated with proteinuria even in patients with MCNS, which is thought not to cause podocyte depletion. These results suggest that massive proteinuria itself may have temporary podocyte detachment and persistence of which may lead to podocyte loss. By contrast, U-sup PCX protein was increased in subepithelial dense deposition-type LN and MN compared with the controls. Previous reports have also shown that u-sup PCX protein is high in MN and LN class V (36).

Hara et al. reported that the u-sup PCX protein does not originate from cell debris of the detached podocyte, but is thought to be a vesicle structure derived from apical cell membrane shedding from microvilli of the injured podocyte (29, 36). In idiopathic MN, antibodies such as anti- phospholipase A2 receptor 1 (PLA2R1) and anti-thrombospondin type-1 domain-containing 7A (THSD7A) have been reported to be involved in the pathogenesis of the disease. It is thought that immune complexes containing these antibodies are formed in subepithelium and activate complement, resulting in podocyte damage and the development of MN (37, 38). We suspect that immune complexes deposited in the subepithelial region cause podocyte injury via complement activation, resulting in vesicle shedding from microvilli and high levels of u-sup PCX protein in MN and LN, but when cell death does not occur (mild podocyte loss), u-sed pod mRNA excretion does not increase. The detailed mechanism of podocyte damage by complement activation in subepithelial dense deposit-type glomerulonephritis requires further investigation in recently developed PLA2R and THSD7A-
related nephropathy models (39, 40). Furthermore, patients with MCNS have massive proteinuria, as do those with MN, but u-sup PCX protein was not increased in this study. These results support the above concept and indicate that u-sed Pod mRNA excretion and u-sup PCX protein could provide different information.

A limitation of this study is that urinary podocyte marker measurements were based on a single urine spot and a small sample size. Serial urinary podocyte markers would be more useful and reliable. In addition, many of the patients were transferred to other hospitals, thus being lost to follow-up. Few patients required renal replacement therapy during the observation period, hampering analysis of their prognosis. Therefore, a long-term prognostic study is required. Despite this limitation, we found that urinary podocyte biomarkers could be useful for monitoring the activity of glomerular diseases.

In summary, we demonstrated that u-sed pod mRNA excretion was increased in multiple glomerular diseases and that u-sup PCX protein was increased in MN and LN. Furthermore, a high level of u-sed Pod mRNA excretion was associated with severe proliferative glomerular diseases, such as IgAN with extracapillary proliferative lesions, Cres GN and LN class IV, and a high level of u-sup PCX protein was associated with subepithelial dense deposit-type GN. These data suggest that urinary sediment podocyte mRNA biomarkers and urinary supernatant podocalyxin protein have potential as diagnostic and disease activity markers in glomerular diseases.
Disclosures

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Authors’ Contributions

Akihiro Fukuda: Data curation; Formal analysis; Funding acquisition; Investigation; Project administration; Writing - original draft. Akihiro Minakawa: Data curation; Formal analysis. Yuji
Sato: Data curation; Formal analysis. Hirotaka Shibata: Data curation; Formal analysis. Masanori Hara: Conceptualization; Supervision. Shouichi Fujimoto: Conceptualization; Project administration; Supervision; Writing - review and editing. All authors approved the final version of the manuscript.

**Supplemental Material**

Supplemental Figure S1. Relationship between urinary podocyte markers and proteinuria in patients with MCNS.

Supplemental Table S1. Clinical parameters, urinary podocyte markers, with or without relapse, and use of immunosuppressive drugs in patients with MCNS.
References


Table 1. Clinical profiles of the patients with kidney disease and healthy controls.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 12)</th>
<th>Minor (n = 15)</th>
<th>MCNS (n = 15)</th>
<th>MN (n = 15)</th>
<th>IgAN (n = 60)</th>
<th>Cres GN (n = 19)</th>
<th>LN (n = 10)</th>
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<td>18/20</td>
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<td>SBP (mmHg)</td>
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<td>119 ± 24</td>
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<td>DBP (mmHg)</td>
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<td>72 ± 14</td>
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<td>71 ± 11</td>
<td>74 ± 11</td>
<td>76 ± 11</td>
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<td>Serum Alb (g/dL)</td>
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Table 2. Clinical parameters, urinary podocyte markers, and areas of dense deposition in patients with LN

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<th>SBP (mmHg)</th>
<th>DBP (mmHg)</th>
<th>TP (g/dL)</th>
<th>Alb (g/dL)</th>
<th>U-Pro/Cr (g/gCre)</th>
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<th>logPC</th>
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*, **, -, no; +/-, partially weak; 1+, weak; 2+, intermediate; and 3+, strong
Table 3. Clinical parameters, urinary podocyte markers, and classification of histological stage in patients with MN

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<th>DBP (mmHg)</th>
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<th>Alb (g/dL)</th>
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Figure Legends

Figure 1. Relationship between urinary podocyte markers and proteinuria. (A) Relationship between urinary sediment podocyte (podocin) (u-sed Pod) mRNA excretion and urinary supernatant podocyte (podocalyxin) (u-sup PCX) protein. (B) Relationship between u-sed Pod mRNA excretion and proteinuria. (C) Relationship between u-sup PCX protein and proteinuria. U-sed pod mRNA excretion was significantly correlated with u-sup PCX protein ($r = 0.37$, $p < 0.001$). U-sed pod mRNA excretion and u-sup PCX protein were significantly correlated with u-PCR ($r = 0.53$, $p < 0.001$ and $r = 0.35$, $p < 0.001$, respectively).

Figure 2. Urinary podocyte biomarkers and proteinuria in glomerular diseases. (A) Proteinuria in glomerular diseases. (B) Urinary sediment podocyte (podocin) (u-sed Pod) mRNA excretion in glomerular diseases. (C) Urinary supernatant podocyte (podocalyxin) (u-sup PCX) protein in glomerular diseases. Proteinuria was significantly increased in all of the glomerular diseases. U-sed pod mRNA excretion was also significantly increased in all of the glomerular diseases compared with controls, and further increased in IgAN, Cres GN and LN, whereas u-sup PCX protein was significantly increased only in MN and LN compared with the controls. *$p < 0.05$ and **$p < 0.01$ vs. control, assessed by Kruskal–Wallis test followed by Dunn test.
Figure 3. Relationship between urinary podocyte markers and histological findings. (A) U-sed Pod mRNA excretion and u-sup PCX protein in the Oxford IgAN histological classification [(M0: n = 35, M1: n = 25, E0: n = 25, E1: n = 35, S0: n = 26, S1: n = 34, T0: n = 50, T1 and 2: n = 10, C0: n = 31, C1 and 2: n = 29]. U-sed Pod mRNA excretion, but not u-sup PCX protein, was significantly increased in the presence of extracapillary proliferative lesions. (B) Relationship between u-sed Pod mRNA excretion and rate of crescent formation in Cres GN. (C) Relationship between u-sup PCX protein and rate of crescent formation in Cres GN. U-sed Pod mRNA excretion, but not sup-PCX protein, was significantly correlated with the rate of crescent formation (U-sed Pod mRNA excretion: n= 17, r = 0.56, p = 0.02; U-sup PCX protein: n = 17, r = -0.12, p = 0.65). *p < 0.05 by Mann–Whitney U test.

Figure 4. Urinary podocyte markers by histopathological classification in patients with LN and MN. (A) Urinary sediment podocyte (podocin) (u-sed Pod) mRNA excretion by histopathological classification in patients with LN. (B) Urinary supernatant podocyte (podocalyxin) (u-sup PCX) protein by histopathological classification in patients with LN. U-sed pod mRNA excretion was increased in class IV, and u-sup PCX protein was increased in subepithelial dense deposit-type LN. (C) Urinary sediment podocyte (podocin) (u-sed Pod) mRNA excretion by histopathological stage in patients with MN. (D) Urinary supernatant podocyte (podocalyxin) (u-sup PCX) protein by
histopathological classification in patients with LN. There was no difference in the podocyte markers according to classification stage (Churg’s stage classification).

**Figure 5. Urinary podocyte markers by histopathological type in patients with IgAN, Cres GN, MN and LN.** (A) Urinary sediment podocyte (podocin) (u-sed Pod) mRNA excretion by histopathological type (mesangial proliferative type, extracapillary proliferative type, and epithelial dense deposit type) in patients with IgAN, Cres GN, MN and LN. (B) Urinary supernatant podocyte (podocalyxin) (u-sup PCX) protein by histopathological type (mesangial proliferative type, extracapillary proliferative type, and epithelial dense deposit type) in patients with IgAN, Cres GN, MN and LN. U-sed pod mRNA excretion was increased in the extracapillary proliferative type, and u-sup PCX protein was increased in the epithelial dense deposit type. *p < 0.05 and **p < 0.01 vs. controls, by Kruskal–Wallis test followed by Dunn test.
Figure 1

A. U-sed pod mRNA vs U-sup PCX protein

B. U-sed pod mRNA vs proteinuria

C. U-sup PCX protein vs proteinuria
Figure 2

A

Proteinuria

Urinary protein: creatinine ratio (log g/gCr)

Control  Minor  MCNS  MN  LN  IgAN  CreGN

B

U-sed pod mRNA

Urinary podocin mRNA: creatinine ratio (log W/gCrX10^-7)

Control  Minor  MCNS  MN  LN  IgAN  CreGN

C

U-sup PCX protein

Urinary podocalyxin protein: creatinine ratio (ng/μmolCr)

Control  Minor  MCNS  MN  LN  IgAN  CreGN
Figure 3

IgA nephropathy

A

Mesangial hypercellularity

U-sed Pod mRNA vs U-sup PCX protein

Endocapillary hypercellularity

U-sed Pod mRNA vs U-sup PCX protein

Segmental glomerulosclerosis

U-sed Pod mRNA vs U-sup PCX protein

Tubular atrophy / interstitial fibrosis

U-sed Pod mRNA vs U-sup PCX protein

Extracapillary lesion (Crescent formation)

U-sed Pod mRNA vs U-sup PCX protein

Crescentic glomerulonephritis

B

U-sed pod mRNA vs Rate of Crescent formation

U-sup PCX protein vs Rate of Crescent formation
**Lupus nephritis**

A. **U-sed pod mRNA**

B. **U-sup PCX protein**

**Membranous nephropathy**

C. **U-sed pod mRNA**

D. **U-sup PCX protein**
Figure 5

A  U-sed pod mRNA

Mesangial Proliferative type  Extracapillary Proliferative type  Subepithelial DD-type

N.S  **  N.S

B  U-sup PCX protein

Mesangial Proliferative type  Extracapillary Proliferative type  Subepithelial DD-type

N.S  **

Urinary podocin mRNA: creatinine ratio \( \log M/g \times 10^{-12} \)

Urinary podocalyxin protein: creatinine ratio \( \log ng/\mu mol Cr \)