

RESEARCH ARTICLE

Elevated Levels of Urinary Podocyte-Derived Microparticles in Nephrotic Syndrome

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Abstract

BACKGROUND: Nephrotic syndrome (NS) is the most common glomerular disease in childhood. The proposed hypothesis for the pathogenesis of this disease has changed over time, from immune dysregulation theory and systemic circulating factors theory, to the growing recognition of podocytopathies' role. The existence of podocytopathies is usually examined by using podocyte-derived microparticles (MPs), such as nephrin, podocin, and podocalyxin (PCX). Therefore in this study, the difference between nephrin, podocin, and PCX expressions in NS children and healthy children was investigated.

METHODS: An observational cross-sectional study was conducted, involving 33 children with NS and 22 age-matched healthy children as controls. Urine samples were collected from each subject in the early morning, before being processed and incubated with antibodies to detect

nephrin, podocin, and PCX. The processed samples were then analyzed with flow cytometer methods.

RESULTS: NS subjects had significantly higher expression of all three urinary podocyte-derived MPs compared to the control subjects. Nephrin, podocin, and PCX showed good discrimination in NS subjects with the area under curve (AUC) of 0.895, 0.849, and 0.728, respectively.

CONCLUSION: This study revealed the differential expression of podocyte proteins in NS subjects compared to healthy controls. This supports the role of podocytopathies in the pathogenesis of NS. Therefore, nephrin, podocin, and PCX might have potentials to be future non-invasive diagnostic tools in glomerular disease.

KEYWORDS: nephrin, nephrotic syndrome, podocalyxin, podocin, podocyte, urinary microparticle

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Introduction

Nephrotic syndrome (NS) is the most common glomerular kidney disease in children, which is characterized by clinical presence of proteinuria, hypoalbuminemia, and edema. The global estimated incidence of NS is 1.15-16.9 cases per 100,000 children annually, varying by race and

ethnicity.(1) According to its underlying causes, there are congenital NS, idiopathic/primary NS, and secondary NS, where idiopathic NS is the most prevalent one to occur in children.(2) The detailed pathogenesis and etiology of idiopathic NS remain elusive. Studies revealed idiopathic NS might be associated with immune dysregulation, systemic circulating factors abnormalities, and the growing recognition of podocytopathies role.(1,2)

Podocytes are visceral epithelium cells that configure the third layer of the filtration barrier system of glomeruli. The innermost layer is the fenestrated capillary endothelial lining, and the second in the middle is the glomerular basement membrane (GBM).(3) Podocytes are very specialized, well-differentiated cells, and thus have a very low rate of turnover.(4) Morphology of a mature cell mimics that octopus' structure, with 3 separate structural and functional elements: a large cell body, major primary processes, and foot processes that interdigitate finely with one another of the neighboring cells. These interdigitating foot processes were linked with a specialized cell junction with signaling properties called the slit diaphragm. The space provided in between the net of interdigitating processes serves as ultra-filtration pores for urine formation.(5)

Histopathological studies of kidney biopsy have shown evidence of impairments in both the quantity and quality of podocytes in various glomerular diseases. Characteristic changes in the morphology of injured podocytes can only be observed by electron microscopy, which is not available in most centers in our country. Those abnormalities include podocyte foot effacement, loss of anion charge, apoptosis, and shedding of podocytes into urine.(6,7) Podocytopeny can also manifest as dedifferentiation of cells, by which the cells grow reversely from the highly structured podocytes to a less differentiated mesangial tissue with a simpler structure.(8) In all types of cells, during the early stage of apoptosis or cell injury, cell membranes will form outward budding and blebbing, subsequently release microparticles (MPs). MPs are small membrane, spherical-shaped extracellular vesicles of <1 mm in size, containing cell bioactive molecules such as cellular proteins, RNA, and mRNA. MPs are involved in intercellular interactions and biological activities associated with inflammation and immune response.(9,10) Thus, MPs may represent the pathological process of podocyte damage and apoptosis in glomerular diseases.(9,11)

The MPs included in this study are nephrin, podocin, and podocalyxin (PCX). They are the most-studied podocyte proteins, probably because they exist on the outermost surface of a podocyte cell body, and thus act as the most adjacent to the urinary space. PCX is located at the apical membrane providing the anionic characteristic to podocyte, while nephrin and podocin regulate the slit diaphragm.(5) To date, published work on absolute quantified values of podocyte-derived MPs in urine of NS children has been limited. Therefore in this study, the difference between urinary nephrin, podocin and PCX MPs expressions in NS children and healthy children was investigated.

Methods

Study Design and Subjects Recruitment

This was an observational cross-sectional study involving children aged 2–18 years old as subjects, which was conducted at Cipto Mangunkusumo Central General Hospital, Jakarta, Indonesia, in 2022. All the protocols and procedures in this study were approved by the Ethics Committee of the Faculty of Medicine, Universitas Indonesia - Cipto Mangunkusumo Central General Hospital (No. KET-854/UN2.F1/ETIK/PPM.00.02/2021). Informed consent was obtained from the subjects' parents. Total 33 NS children and 22 age-matched healthy children (as controls) were recruited, based on the minimal sample size calculated with following formula:

$$n_1 = n_2 = 2 \left(\frac{z\alpha + z\beta}{x_1 - x_2} \right)^2$$

All NS subjects enrolled in this study were patients with active episodes of NS, whether it was an initial manifestation or a relapse, and had not received the definitive therapy yet. The active episode of NS was identified by nephrotic-range proteinuria, defined as urine protein-creatinine ratio (uPCR) value ≥ 2000 mg/g of creatinine. The glomerular filtration rate (GFR) was estimated using Schwartz formula.(12) All our NS subjects had a preserved GFR by the time of sampling. Subjects with comorbidities and deteriorated renal function were ruled out. Control subjects were healthy children <18 years old with no history of chronic or congenital disease, no proteinuria, and were born with normal birth weight. Phlebotomy was not performed in healthy subjects; creatinine and estimated GFR (eGFR) values were thus considered normal.

Detection of Microparticle Podocytes in Urine

Midstream urine samples were collected from each subject in the early morning. After collection, the morning urine was processed within 4 hours. Ten mL of homogenized urine was centrifuged for 5 minutes at 2,000 rotation per minute (rpm) of speed at room temperature. The supernatant was transferred separately and then centrifuged again at 4000 rpm for 20 minutes, 4°C of temperature. A hundred μ L of the uppermost part of the solution was transferred to a TruCount® tube (BD Bioscience, San Jose, CA, USA), then added 1.25 μ L of mouse anti-nephrin monoclonal antibody Alexa Fluor® conjugated (Cat. #sc-377246 AF64, Santa Cruz Biotechnology, Dallas, TX, USA), mouse anti-human PCX monoclonal antibody FITC conjugated (Cat.

#374304, Biolegend, San Diego, CA, USA) and mouse anti-podocin monoclonal antibody PE conjugated (Cat. #sc-518088 PE, Santa Cruz Biotechnology), respectively. It was then incubated for 15 minutes in a dark condition and room temperature. Five hundred μL of FACSLyse[®] solution (BD Bioscience) was added followed by 15 minutes incubation. Samples were analyzed by flow cytometer FACSCanto[®] with FACSDiva[®] software (BD Bioscience). Forward scatter (FSC) and side scatter (SSC) logarithmic settings were used. MPs were identified based on the standard size of Megamix[®] (BioCytex, Marseille, France) to include particle sized 0.5-0.9 μM . The absolute count of MPs/ μL was obtained by calculating the MP events to reference beads events of TruCount[®] tube. The value of MPs/ μL in urine were normalized to the subjects' urine creatinine secretion. The MP:creatinine ratio results were then displayed as MP/mg Cr.

Statistical Analysis

Statistical analyses were performed using Statistical Package for Social Sciences version 20.0 for Windows (IBM Corporation, Armonk, NY, USA). The normality test was done with the Kolmogorov-Smirnov test. A nonparametric Mann-Whitney U-test was used to compare differences in levels of nephrin, podocin, and PCX MPs between active NS subjects and controls. Correlations between levels of proteinuria and urinary podocyte MPs were determined using a non-parametric Spearman test. A p -value <0.05 was considered statistically significant. Receiver operating characteristic (ROC) curves were calculated to evaluate the discrimination value of the MPs.

Results

The baseline characteristics of all subjects were summarized in Table 1. Of all 33 active NS subjects, 9 were initial NS manifestations, while the other 24 subjects were in relapse

episodes. Serum creatinine and eGFR of NS subjects were all in the normal range.

In our study, all NS and control subjects' urine samples indicated positive expression of podocyte-derived MPs (Figure 1). The 22 control subjects had consistently low MP expression of nephrin, podocin, and PCX, compared to NS subjects, and were statistically significant ($p<0.01$) (Table 2, Figure 2). Modest positive correlations were shown between uPCR and the three biomarkers and were statistically significant (Figure 3). The urinary biomarkers also showed a positive correlation with one another (Figure 4). No statistical correlations were found between urinary podocyte MPs with the subjects' age, disease duration nor eGFR.

ROC analysis and area under the curve (AUC) for each biomarker were calculated to evaluate the discrimination value between NS subjects and control subjects. (Figure 5, Table 3). All three urinary MPs had AUC >0.50 and had statistical significance with $p<0.01$. The most acceptable cut-off found for MPs was indicated with sensitivity and specificity stated (Table 3).

Discussion

This study showed that the levels of podocyte-derived MPs of nephrin, podocin, and PCX were all significantly elevated in urine samples of NS children compared to healthy controls with acceptable discrimination of AUC. We tried to reduce confounding factors by enrolling the patients in the newly recognized active state of NS with preserved eGFR only, which had not received steroids yet at the time of the sampling. All ages between 2-18 years were covered and did not differ between the two groups.

Podocyte lining is responsible for both mechanical and functional fashions of glomerular filtration. Between each interdigitating pedicles, lay a narrow slit diaphragm allowing materials with a specific size to pass into urine

Table 1. Demographic data of active NS subjects and control subjects.

Variables	Active NS (n=33)	Control (n=22)	p-value
Age (years), mean \pm SD	7.80 \pm 4.67	9.41 \pm 4.75	0.293 ^a
Sex (male/female), n	17/16	8/14	0.407 ^b
Disease duration (years), mean \pm SD	2.56 \pm 3.07	ND	-
Serum creatinine (mg/dL), mean \pm SD	0.42 \pm 0.19	ND	-
eGFR (mL/min/1.73 m ²), mean \pm SD	128.99 \pm 39.68	ND	-
uPCR (mg/g), mean \pm SD	11,230.00 \pm 5,232.55	199.25 \pm 55.48	0.000 ^{a*}

^aanalyzed with Mann-Whitney test; ^banalyzed with Chi-square analysis. *is considered statistically different if $p<0.05$. ND: not determined.

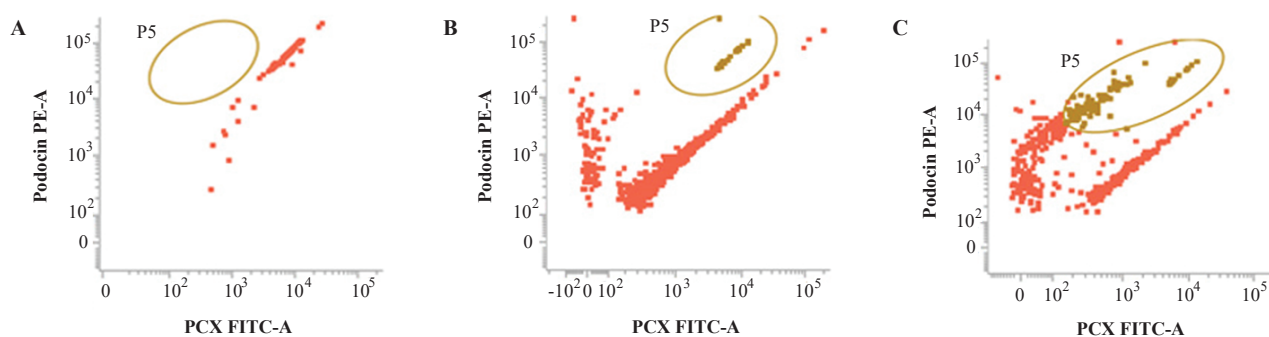


Figure 1. Flowcytometry analysis of podocyte-derived MPs. A: unstained, B: control, C: NS.

lumen.(6,13) PCX is an anionic apical transmembrane protein of the podocyte, served as the negative charge of the podocyte. It is known to have a role in protein trafficking, ion transport, and signaling, also associated with actin-binding function, regulating the cytoskeleton and morphology of the pedicles.(14) Loss of PCX causes immature morphology of the podocyte body, with poor differentiation of the pedicle processus and loss of negative charge.(15) Nephtrin is a transmembrane protein on the lateral surface of the pedicle processus, served to maintain the scaffolding of the slit diaphragm and mediating cell signaling pathways. Podocin is a membranous protein on the lateral surface of the foot processus. It is able to communicate with nephtrin and regulate the podocyte structure. Nephtrin and podocin are expressed on the extracellular surface and intercellular cytoplasm of the cytoskeletal pattern.(16,17)

Other glomerular diseases such as NS, Immunoglobulin A nephropathy (IgAN), lupus nephritis (LN), and diabetic nephropathy, immunofluorescence staining of kidney biopsies revealed impaired expressions of podocyte protein biomarkers, such as nephtrin, podocin, and PCX. These studies showed decreased expression, uneven distribution, and changed patterns of podocyte biomarkers compared to control.(18-21) Study demonstrated that podocytes are shed into urine in the form of both a whole cell and granular fragments using PCX-antibody in the immunofluorescence staining. Both groups of control subjects and active glomerulonephritis patients showed positive urinary expression of these biomarkers, with elevated protein in

the disease group. This study provided convincing evidence for a correlation between the microvillous structure on the podocytes surface in the kidney and the vesicle-like structure in urine sediments.(7)

Western blot study presented that band precipitation of podocyte biomarkers of nephtrin was thicker in various glomerulopathies compared to healthy control. Podocyte cell count using anti-PCX-stained immunofluorescence was higher in the disease group, and the nephtrin enzyme-linked immunosorbent assay (ELISA) level of 10 healthy subjects was undetected.(22) Also with immunofluorescence staining, previous studies showed similar results with no podocyte cell count detected in 20 healthy controls.(23,24) expression of PCX was also evident in LN using mRNA detection (normalized to β -actin) (25) and ELISA (26), as well as in diabetic nephropathy disease using mRNA quantification (27), with all displayed positive expression of podocyte PCX in controls. Another study presented an AUC of 0.821 in discriminating urinary PCX levels using ELISA in the glomerulopathies group from the control.(28) Elevated urinary nephtrin and podocin mRNA expression in various glomerulopathies were also found in several studies.(29-33) Levels of podocyturia and proteinuria were positively correlated.(29,30)

There are only a few studies we could find that focused on the pediatric population, especially in NS disease. Studies in glomerulonephritis and NS children using immunofluorescence and ELISA methods showed that PCX levels in both the sediment and supernatant urine were higher

Table 2. Levels of podocyte-derived MPs in active NS subjects and control subjects.

Podocyte-derived MPs	Active NS (n=33)	Control (n=22)	<i>p</i> -value
Nephtrin ($\times 10^5$ MP/mg Cr)	13 (9.5 - 19.5)	5.5 (4 - 7)	0.000*
Podocin ($\times 10^5$ MP/mg Cr)	88 (20.5 - 1464.5)	14 (9 - 30.5)	0.000*
PCX ($\times 10^5$ MP/mg Cr)	12 (8 - 21)	7.5 (4 - 11.5)	0.004*

Data presented in median (IQRs). IQR: Interquartile range. *is analyzed with Mann-Whitney test, and considered statistically different if $p < 0.05$.

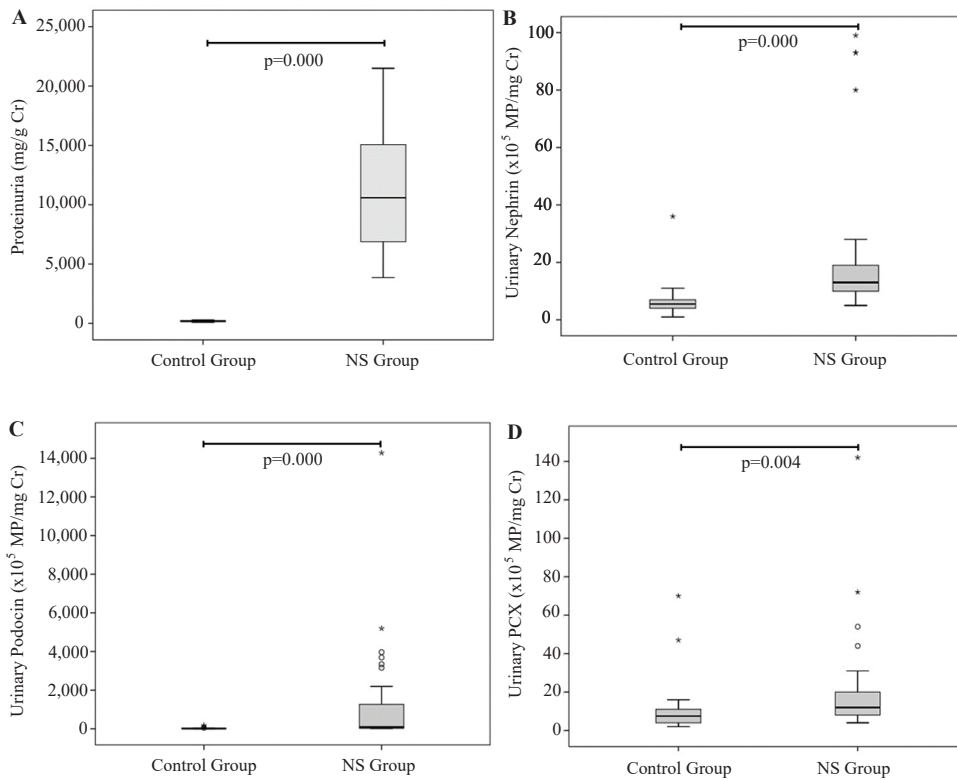


Figure 2. The levels of absolute proteinuria and urinary podocyte MPs in active NS subjects (n=33) vs. the control subjects (n=22). A: Proteinuria (is displayed as uPCR); B: Urinary nephtrin; C: Urinary podocin; D: Urinary PCX.

in the study group compared to the control. Both studies recruited quite a large population of around 200 children subjects.(34,35) A study that focused only on NS children was done by using a turbidimetric immunoassay (TIA) and showed elevated PCX levels in active NS compared to NS in remission and control group.(36)

Previous studies showed that renal function may be associated and correlated with levels of urinary podocyte expression.(33,37,38) Our study, for the first time, tried to investigate the MP expressions in primary NS pediatrics with preserved renal function. As we discussed earlier, some studies with the immunofluorescence method showed no

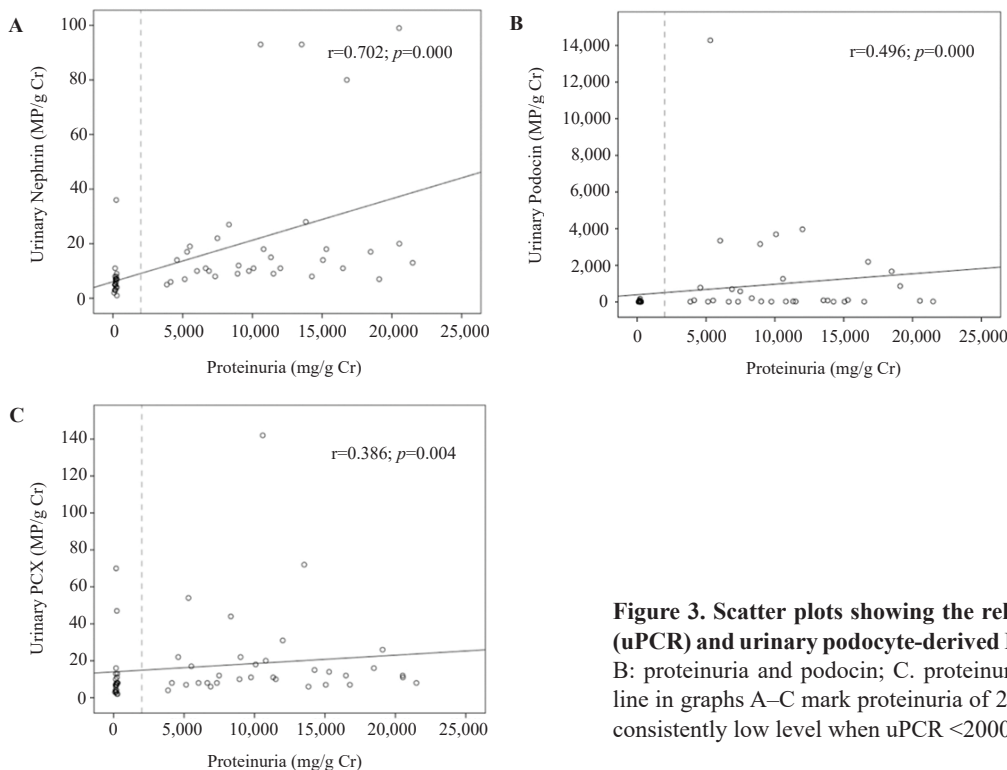


Figure 3. Scatter plots showing the relationship between proteinuria (uPCR) and urinary podocyte-derived MPs. A: proteinuria and nephtrin; B: proteinuria and podocin; C: proteinuria and PCX. Vertical grey dash line in graphs A–C mark proteinuria of 2000 mg/g Cr. Graph A showed a consistently low level when uPCR <2000 mg/g.

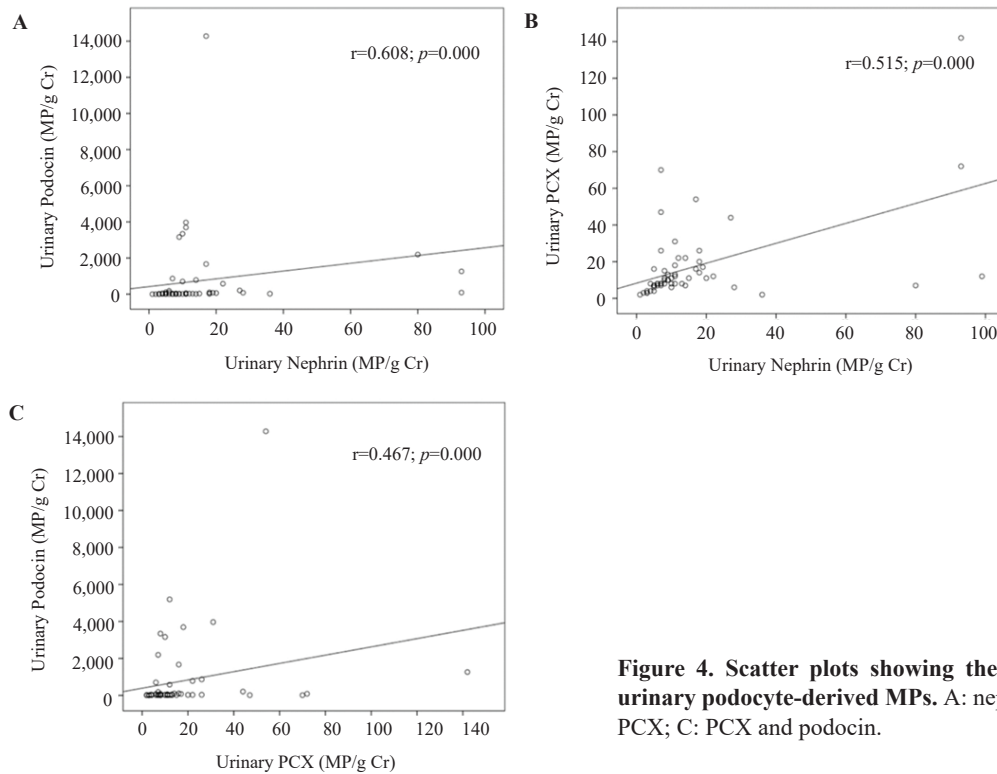


Figure 4. Scatter plots showing the relationship between levels of urinary podocyte-derived MPs. A: nephrin and podocin; B: nephrin and PCX; C: PCX and podocin.

expression of podocyte count in control patients.(23,39) A different result was revealed by mRNA and ELISA studies where they all showed positive expression of podocyte-derived MPs in healthy subjects.(25-27,29-32).

Similar to those mRNA and ELISA studies, our study confirmed that podocyte MPs are present in urine, even in healthy individuals. Previous studies showing negative expression in healthy subjects used different modalities and only worked with the sediment as urine specimen, (26-28,30-33) while our study analyzed the urine supernatant. The small sample size and using only the urine sediment might

explain the negative expression with immunofluorescence microscopy from previous studies.(23,24) Moreover, this study aimed to observe the expression of MPs, and not the podocyte as a whole cell. Microparticles are vesicles shed by podocyte cells, so its expression in urine should not be immediately concluded as podocyte death/loss in glomerulus. In one immunofluorescence study, podocytes culture revealed no growth from the healthy subjects' urine.(23) This may indicate that the expression of podocytes in the control urine are indeed not viable, thus not excreted as a whole living cell. Previous publications displaying urinary podocyte MP expressions in NS are scanty, especially in pediatric population. In another study using PCX-antibody, the IQR in healthy adult subjects ranged between $0.46\text{--}2.77 \times 10^6/\text{mL}$ (median $1.05 \times 10^6/\text{mL}$) (40), while in our study, PCX IQR ranged between $8\text{--}21 \times 10^5/\text{mg}$ corrected to creatinine (median $12 \times 10^5/\text{mg Cr}$).

We found significantly higher urinary podocyte-derived nephrin, podocin, and PCX, levels in the NS group compared to the control. Although the difference is statistically significant, we noticed overlapping values between the control and NS group (Figure 2) and found difficulty in determining the discrimination point between the two groups. A similar graph plot was also observed in other previous studies.(11,25,33) Our model displayed sensitivity and specificity ranging from 0.6–0.8 for each MP (Table 3). A positive correlation was found between

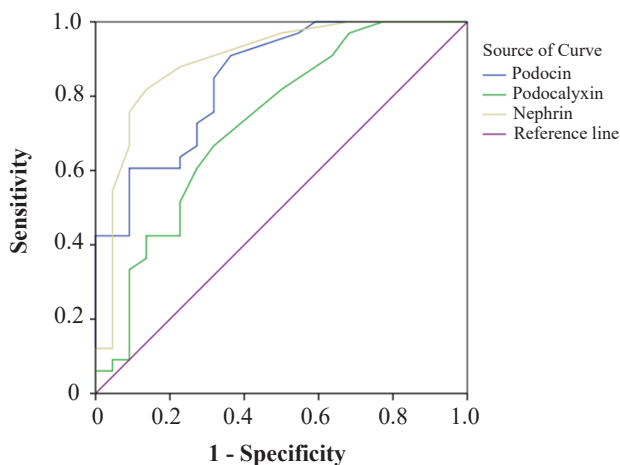


Figure 5. ROC analysis of urinary podocyte-derived MPs in discriminating active NS from control.

Table 3. AUC analysis for podocyte-derived MPs.

Microparticle	AUC	Cut-off	Sensitivity	Specificity	p-value
Nephrin (in MP/mg Cr)	0.895	8.5×10^5	0.818	0.864	0.000*
Podocin (in MP/mg Cr)	0.849	16.5×10^5	0.848	0.682	0.000*
PCX (in MP/mg Cr)	0.728	9×10^5	0.667	0.682	0.004*

uPCR and all podocyte-derived MPs ($r=0.702$, 0.496 , and 0.386 for nephrin, podocin, and PCX, respectively). In another study investigating systemic lupus erythematosus and LN patients, urinary PCX MPs also showed a significant correlation with proteinuria ($r=0.674$, $p<0.0001$) and serum albumin ($r=-0.764$, $p<0.0001$).⁽¹¹⁾ Elevated levels of nephrin and podocyte counts were observed in group of FSGS compared to mesangial proliferative glomerulonephritis (MPGN) and minimal change disease (MCD) groups.⁽²⁸⁾

Conclusion

The expression of urinary podocytes-derived nephrin, podocin, and PCX microparticles in NS patients is found higher when compared to healthy controls. This supports the role of podocytopathies in the pathogenesis of NS. Further studies are required to investigate its potential as a noninvasive diagnostic tool to differentiate types of glomerulopathies, *e.g.*, histopathology types in NS.

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Authors Contribution

ELH, SOP, PPT, and BS were involved in the conception, research, data acquisition and analysis/interpretation. ELH, PPT, ORR, DS, and DW collaborated in the preparation/revision of the submitted manuscript. ELH and DW ensured a proper explanation to possible questions that could be raised regarding accuracy and scientific integrity of the submitted. All authors took parts in giving critical revision of the manuscript.

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