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ARTICLE

Nephronectin (NPNT) and the prediction of nephrotic syndrome response to steroid treatment

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Abstract

Steroid-resistant nephrotic syndrome represents about 10–20% of pediatrics' nephrotic syndrome. The regeneration of glomerular barrier seems pivotal for cessation of proteinuria. Nephronectin (NPNT) plays a major role in nephrogenesis, signal transduction, and epithelial–mesenchymal interactions. This study aims to preliminary assess NPNT as potential noninvasive biomarker of glomerular regeneration and its ability to identify steroid resistance. In this case control study, 80 retrospectively selected patients with nephrotic syndrome were enrolled in addition to 40 healthy controls. Forty patients were steroid sensitive (SSNS) and the other 40 patients were steroid-resistant (SRNS), NPTN concentration was measured using ELISA and NPNT mRNA expression was assayed using real-time PCR. NPTN concentrations were significantly higher in SSNS than both SRNS and controls (The means were 4.64 ± 3.05, 0.69 ± 0.44, and 1.63 ± 0.59, respectively). Moreover, NPTN concentrations were significantly lower in SRNS than controls. NPTN was significantly overexpressed in SSNS compared to both SRNS and controls (the means were 10.82 ± 7.39, 1.19 ± 0.94, and 1.04 ± 0.10, respectively) with no statistically significant difference between SRNS and controls. ROC curves analysis showed that both NPNT expression and NPNT serum level are of promising diagnostic performance (ROC_{AUC} 0.948 and 0.896, respectively). Regression analysis showed that both NPNT expression and NPNT serum level can be independent predictors of steroid resistance. The present study shows for the first time an enhanced expression of NPNT in steroid-sensitive nephrotic syndrome patients suggesting NPNT as a marker of glomerular regeneration. Also, serum NPNT can be a useful noninvasive biomarker of steroid resistance.

Introduction

Nephrotic syndrome (NS) is a syndrome characterized by increased glomerular filtration barrier permeability. It classically presents with heavy proteinuria (Urine protein excretion > 50 mg/kg/day or a spot urine sample with albumin/creatinine ratio (UACR) greater than 3 g/g creatinine), hypoalbuminemia (serum albumin < 3 g/dL), edema, and hyperlipidemia. The majority of children who present with NS have minimal change disease (MCD) which is generally responsive to steroid therapy [1]. Hence, empirical steroid therapy is given to children who present with idiopathic NS. However, ~10–20% of patients fail to respond to initial steroid treatment and are termed to have steroid-resistant nephrotic syndrome (SRNS) [2]. The consensus guidelines are to define steroid resistance by persistence of proteinuria after 6 weeks of steroid treatment with daily prednisolone 60 mg/m, twice a day [3].

SRNS is a chronic progressive disorder affecting about 10% of all children with NS. Long term aggressive therapy with combinations of steroids, cyclosporine and alkylating agents like cyclophosphamide may cause complete or partial remission in 20–80% patients. Also, non-specific renal protective agents such as the ACE (angiotensin converting enzyme) inhibitors, angiotensin-2 receptor blockers, and anti-lipid agents can help to retard disease progression [4].

The glomerular filtration barrier is composed of inner endothelial layer and outer podocyte layer with the basement membrane and extracellular matrix (ECM) in-between. The interaction between those cells and the

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surrounding ECM is essential to maintain the barrier functions of the glomerulus [5]. This interaction is mediated through integrin receptors that are trans-membrane glycoproteins of heterodimeric structure consisting of α and β subunits [6].

Nephronectin is a tissue-specific and developmentally regulated major ECM protein of 70–90 kDa, identified as integrin α8β1 ligand involved in kidney development [7–9]. The physiological functions of nephronectin is poorly understood, it is thought to play a role in epithelial–mesenchymal interactions via binding to α8β1 integrin, thus transmitting signals across the basement membrane [7, 10].

Other integrin ligands, including fibronectin and osteopontin, fail to compensate for nephronectin deficiency in the developing kidneys suggesting that nephronectin is a mandatory α8β1 integrin ligand required for epithelial–mesenchymal interactions. Moreover, nephronectin is the preferred α8β1 integrin ligand and the C-terminal side of its RGD motif serves as a synergistic site ensuring a specific 100-fold high affinity binding of nephronectin to α8β1 integrin [11, 12].

Podocytes are highly specialized post-mitotic terminally differentiated epithelial cells with numerous foot processes (FP) that have interdigitating pattern. Those FP are adherent to the outer layer of the glomerular basement membrane (GBM) while the cell body floats in Bowman's space [13].

FP integrity is mandatory to maintain the glomerular filtration barrier. Intact FP are fixed to the GBM by various integrin dimers and by α- and β-dystroglycans. Deletion of any of the integrins from podocytes has fatal consequences, most severe after deletion of α3 or β1-integrin [13].

During glomerular injury, podocytes undergo several changes in their shape. The FP loose their regular interdigitating pattern (effacement) followed by loss of the space beneath the podocyte cell body then, finally by rearrangement of the cytoskeleton. This rearrangement includes formation of cytoskeletal mat closely apposed to the GBM, suggesting reorganization of the podocyte–GBM interactions [13].

FP effacement is the most prominent and the most enigmatic structural event occurring in many glomerulopathies. And the degree of proteinuria is dependent on the degree of FP effacement [14].

Finding novel biomarkers that can assess glomerular regeneration in response to steroid treatment will help to predict treatment failure as early as possible, avoid the unnecessary long duration of steroid treatment with its side effects and address the physician to early start other lines of treatment.

This study aimed to investigate nephronectin as a marker of glomerular regeneration in NS patients treated with corticosteroids and its probable use for differencing steroid responders from steroid-resistant patients.

Patients and methods

Study population

This case control study was carried out in the duration from June 2016 to August 2017. The study was conducted on 80 selected patients with NS collected from the pediatrics department, Tanta university hospital, Egypt. Forty healthy controls of matching age and sex were also enrolled from the outpatient Clinique. An informed written consent was obtained from the parents of all participants. The study was approved by the local ethical committee of faculty of medicine Tanta University and the research is complied with Helsinki Declaration.

At the time of diagnosis all patients were subjected to complete history taking with special attention to recent skin and throat infections, fever, drug administration, and any change of urine color. Patients were also subjected to physical examination to document fever, edema, ascites, nutritional status, volume status, and hypertension. The laboratory data including; complete blood count, anti-Sterptolysin O titre (ASOT), Serum Creatinine (Scr), eGFR using Schwartz equation, cholesterol, albumin, 24 h urine protein, complete urine analysis, and UACR were also collected.

Patients characteristics

NS was diagnosed by the triad of heavy proteinuria (>3 gm/day), hypoalbuminemia, and edema. The steroid-sensitive nephrotic syndrome (SSNS) group included 40 patients who responded to steroid treatment within 6 weeks. The SRNS group included 40 patients who showed persistent protienuria after 6 weeks of corticosteroid treatment.

The patients were retrospectively selected upon the outcome of 6 weeks of corticosteroid treatment. All patients received the same dose of corticosteroid for 6 weeks (prednisolone 2 mg/kg/day), ACE inhibitors were only given to patients with hypertension.

Patients with history of infection, high ASOT, hematuria, fever, protein energy malnutrition, and autoimmune diseases were excluded.

Samples collection and biochemical analysis

Blood samples were collected in VACUETTE® Blood Collection Tubes (Greiner Bio-One, Austria). Serum samples were collected in serum separating tubes and separated by centrifugation at 10000× *g* for 10 min. Scr, cholesterol, and albumin were measured using automated chemistry analyzer (Konelab Prime-60i, Vantaa, Finland). NPNT concentration was measured by ELISA. Samples designed for RNA extraction were collected on EDTA. 24 h urine

protein excretion was determined only once at the diagnosis; screened using 3% sulphosalsylic acid, and confirmed by immune-turbidimetric method (BioSystems, Spain) then the patients were followed up by morning sample UACR.

Assay of serum NPNT levels

NPNT concentrations were measured using Enzyme-linked Immunosorbent Assay kit for Nephronectin (USCN life science, cat no. SEH522Hu, Houston, USA) according to manufacturer's instructions.

The kit applies the sandwich immunoassay technique; the provided microtiter plate is pre-coated with an antibody specific to NPNT. Standards and samples were added with the biotin-conjugated anti-NPNT to the microtiter plate wells. After 1 h of incubation at 37°c, all the fluids in the well were discarded, and then the Avidin conjugated to Horseradish Peroxidase (HRP) was added to all wells. The plate was incubated again for another 30 min, and then washed to remove excess unbound biological materials. After TMB substrate solution addition; only the wells containing NPNT, biotin-conjugated antibody, and enzyme-conjugated Avidin exhibited change in color. The enzyme-substrate reaction was terminated by the addition of sulphuric acid stop solution and the color change was measured spectrophotometrically at a wavelength of 450 nm (Tecan Spectra II Microplate Reader, Hombrechtikon, Switzerland).

Curve expert 1.40 software was used to generate the standard curve by plotting the mean optical density (O.D) of each standard against its concentration and drawing the best fit curve. The concentration of NPNT in the samples was then determined by comparing its O.D to the standard curve.

Assay of NPNT mRNA expression levels

Quantitative real-time PCR analysis was used to measure NPNT gene expression. RNA was extracted from 1 ml EDTA blood using Qiamp® RNA Blood Mini Kit (Qiagen GmbH, Germany), according to the manufacturer's instructions. RNA was extracted immediately after sample collection and its concentration and quality were measured using NanoDrop 2000 (Thermoscientific, USA). Three hundred nanograms of RNA were utilized to synthesize cDNA using HiSenScript™ RH [-] cDNA synthesis kit (iNtRON biotechnology, Korea) according to the manufacturer's instructions. cDNA was stored in cryotubes at −80°C till the expression experiment time.

TaqMan real-time quantitative PCR amplification reactions were carried out with step one® Real–Time PCR System (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems, Foster city, California, USA). The reaction mixture contained about 30 ng cDNA, 1 µL Taqman assay®, and 10 µL 2 × TaqMan Buffer® (Applied Biosystems, Foster city, California, USA) in a total reaction volume of 20 µL under standard conditions (initial setup 2 min at 50°C, 10 min at 95°C, 40 cycles of (denaturation for 15 s at 95°C, annealing for 1 min at 60° C).

After validation, GAPDH was used as internal control gene. All reactions were executed in duplicate. In the case of negative control, cDNA was not added. The cycle threshold (CT) values were used for normalized relative quantitation of the NPNT expression. The relative concentration of NPNT gene expression in each sample was calculated on basis of CT corrected by GAPDH expression CT in the same sample using the comparative method formula $2^{-\Delta\Delta}$CT.

Statistical analysis

The data were presented as means ± SD and as frequencies and percentages when appropriate. Kruskal–Wallis test and Chi X2 test were used for the comparison of numerical and non-numerical data, respectively. Receiver operator characteristic (ROC) curves were constructed to assess sensitivity, specificity, and respective area under the curve. The optimum cutoff value was investigated by maximizing both sensitivity and specificity and minimizing the distance from the left upper corner of the ROC curves. Pearson correlation was run to study the correlation between NPNT gene expression, its serum level and the laboratory findings. Regression analysis was performed to assess the studied parameter as potential predictors of steroid resistance. The statistical analysis was performed using IBM SPSS statistics 19 for windows. *P* values less than 0.05 were considered statistically significant.

Results

The study was carried out over 15 months; 80 selected patients with NS were enrolled in addition to 40 healthy controls. Forty patients had SSNS and 40 had SRNS.

In the SSNS group the mean of patients ages was 8.3 ± 3.7, 62.5% of them were males, all of them had generalized edema, and only 2 (5%) of them were hypertensive. In the SRNS group the mean of patients' ages was 6.8 ± 4.2, 40% of them were males, all of them had generalized edema, and 45% of them were hypertensive. In control group, the mean of their ages was 6.3 ± 3.8 and 57.5% of them were males. Comparing the means of age among SSNS, SRNS patients and controls no statistically significant difference was found between them (*P* values

Table 1 Summary of the clinical and laboratory data of the studied groups

	SSNS	SRNS	Controls
Age (mean ± SD)	8.3 ± 3.7	6.8 ± 4.2	6.3 ± 3.8
Sex *n* (%)			
Male	26 (62.5%)	16 (40%)	23 (57.5%)
Female	14 (37.5%)	24 (60%)	17 (42.5%)
Edema *n* (%)	40(100%)	40 (100%)	0(0%)
Hypertension *n* (%)	2 (5%)	18 (45%)[a]	0(0%)
Serum creatinine(mg/dl) (mean ± SD)	0.8 ± 0.28	1.0 ± 0.38[a]	0.8 ± 0.36
Serum albumin (mg/dl) (mean ± SD)	2.21 ± 0.53	2.61 ± 0.32[a]	3.8 ± 0.28
Serum cholesterol (mgldl) (mean ± SD)	312 ± 90.8	438.2 ± 110.3[a]	68.7 ± 21.3
UACR gm/gm (mean ± SD)	4.14 ± 0.79	4.72 ± 0.96[a]	0.107 ± 0.032
eGFR ml/min/1.73 m2 (mean ± SD)	88 ± 13.8	71.3 ± 14.57[a]	84.38 ± 12.1

SSNS steroid-sensitive nephrotic syndrome, *SRNS* steroid-resistant nephrotic syndrome, *UACR* urinary albumin/creatinine ratio, *eGFR* estimated glomerular filtration rate.

[a]Statistically significant form SSNS (*P* value < 0.05)

were > 0.05). The demographic, clinical and laboratory data are all summarized in Table 1.

The comparison of SSNS and SRNS patients' clinical findings showed a statistically significant difference regarding the incidence of hypertension, which was evidently higher in the SRNS group (*P* value < 0.001). Also, comparison of the laboratory findings showed statistically significant differences between SSNS and SRNS regarding serum creatinine, eGFR, albumin, cholesterol, and urinary ACR. *P* values were 0.009, <0.001, 0.001, 0.001, and 0.004, respectively.

Renal biopsies from patients with SRNS revealed that four of them had MCD; the majority (18/40) had focal segmental glomerulosclerosis(FSGS), (12/40) had membranous glomerulonephritis (MN), (5/40) had membranoproliferative glomerulonephritis (MPGN), and one patient showed non conclusive histological findings

Evident higher levels of NPNT mRNA expression were found in the SSNS patients. The means of NPNT gene expression in SSNS, SRNS, and controls were 10.82 ± 7.39, 1.19 ± 0.94, and 1.04 ± 0.10, respectively. The comparison of these means revealed statistically significant differences between SSNS and SRNS and also between SSNS and controls (*P* values were both < 0.001). No significant difference was found between SRNS and the control (*P* 0.322). Moreover, NPTN serum level were evidently higher in the SSNS patients. The means of NPTN serum levels were 4.64 ± 3.05, 0.69 ± 0.44, and 1.63 ± 0.59 in SSNS, SRNS, and controls, respectively. Comparison of these means

Table 2 Case summaries and comparison of NPNT expression and serum level results among the 3 studied groups

	SSNS group (*n* = 40)	SRNS group (*n* = 40)	Control group (*n* = 40)	*P* value
NPNT expression (mean ± SD)	10.82 ± 7.39 [a,c]	1.19 ± 0.94 [b]	1.04 ± 0.10 [b]	<0.001
NPNT serum level (ng/ml) (mean ± SD)	4.64 ± 3.05 [a,c]	0.69 ± 0.44 [b,c]	1.63 ± 0.59 [a,b]	<0.001

[a]*P* value < 0.05 (significant) compared to steroid-resistant nephrotic syndrome (SRNS)

[b]*P* value < 0.05 (significant) compared to steroid-sensitive nephrotic syndrome (SSNS)

[c]*P* value < 0.05 (significant) compared to control

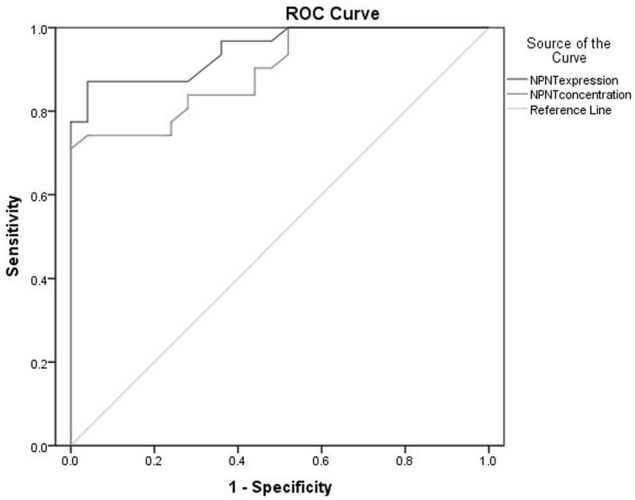

Fig. 1 ROC curves of NPNT expression and NPNT level

showed statistically significant differences between SSNS and SRNS, SSNS, and controls and also between SRNS and the control (*P* values were all < 0.001). (Table 2)

ROC curve analyses were performed to study the diagnostic performance of NPNT expression and NPNT serum level and their ability to differentiate SSNS from SRNS. For the NPNT expression the optimum cutoff was ≥ 2.495 (AUC = 0.948 (0.895–1); 95% CI; sensitivity = 87.1%; specificity = 92%; *P* < 0.001). For NPNT serum level the optimum cutoff was ≥ 1.215 ng/ml (AUC = 0.896 (0.817–0.975); 95% CI; sensitivity = 74.2%; specificity = 92%; *P* < 0.001). (Fig. 1)

Pearson correlation was run (two-tailed) to correlate NPNT expression and serum level to the other laboratory findings of NS such as serum creatinine, albumin, cholesterol, and urine ACR. (Table 3)

For NPNT expression; significant positive correlation was observed between NPNT expression and its serum level ($r = 0.801$, $P < 0.001$), significant negative correlation with urinary ACR was also found ($r = -0.602$, $P = 0.002$), and

Table 3 Correlation matrix of nephronectine (NPNT) expression and its serum level in relation to other variables

		NPNT expression	NPNT serum level	UACR	Serum cholesterol	Serum albumin	Serum createnine	eGFR
NPNT expression	Pearson [*r*]	1	0.801	−0.602	−0.311	0.334	0.018	0.203
	Sig. (*P* value)		0.000[b]	0.02[a]	0.094	0.071	0.913	0.823
NPNT serum level	Pearson [*r*]	0.801	1	−0.403	−0.203	0.152	0.036	0.311
	Sig. (*P* value)	0.000[b]		0.04[a]	0.283	0.422	0.85	0.094
UACR	Pearson [*r*]	−0.602	−0.403	1	0.274	−0.426	0.037	−0.12
	Sig. (*P* value)	0.02[a]	0.04[a]		0.143	0.019[a]	0.847	0.527
Serum cholesterol	Pearson [*r*]	−0.311	−0.203	0.274	1	−0.541	0.168	−0.183
	Sig. (*P* value)	0.094	0.283	0.143		0.042[a]	0.632	0.334
Serum albumin	Pearson [*r*]	0.334	0.152	−0.426	−0.541	1	−0.237	0.248
	Sig. (*P* value)	0.071	0.422	0.019[a]	0.042[a]		0.207	0.193
Serum Createnine	Pearson [*r*]	0.018	0.036	0.037	0.168	−0.237	1	−0.786
	Sig. (*P* value)	0.913	0.85	0.847	0.632	0.207		0.012[a]
eGFR	Pearson [*r*]	0.203	0.311	−0.12	−0.183	0.248	−0.786	1
	Sig. (*P* value)	0.823	0.094	0.527	0.334	0.193	0.012[a]	

NPNT Nephronectin, *UACR* urinary albumin/creatinine ratio, *eGFR* estimated glomerular filtration rate, *r* Pearson correlation coefficient

[a]Correlation is significant at 0.05 (two-tailed)

[b]Correlation is significant at the 0.01 level (two-tailed)

Table 4 Multiple linear regression analysis for NPNT as potential predictors of steroid resistance among nephrotic syndrome patients

	Unstandardized coefficients		Standardized coefficients	*t*	*P* value
	B	Std. Error	β		
NPNT expression	−0.027	0.009	−0.395	−3.010	0.004*
NPNT concentration	−0.068	0.021	−0.408	−3.175	0.003*

* *P* value < 0.05 (significant)

no significant correlation was observed with either serum albumin, serum cholesterol, serum creatinine, or eGFR.

Regarding NPNT serum level, significant negative correlation was found between NPNT serum level and urinary ACR ($r = -0.403$, $P = 0.04$), and no correlation was found with serum albumin, serum cholesterol, or serum creatinine. Moreover, significant negative correlations were found between serum albumin and both serum cholesterol and urinary ACR.

Regression analysis proved that both NPNT mRNA expression and NPNT serum concentration can be independent predictors of steroid resistance among NS patients (*P* 0.004 and 0.003, respectively). (Table 4)

Discussion

Nephronectin is a highly conserved ECM protein that plays critical roles in regulating cell adhesion, differentiation, and survival. It acts as adhesion molecule and is involved in morphogenesis and functions of various tissues, such as the kidneys, liver, and bone [10, 15].

Glomerular filtration barrier repair may include increased expression of the proteins responsible for signal transduction and the interaction between glomerular cells and the surrounding ECM including NPNT.

This work showed that NPNT mRNA expression and NPNT serum levels were significantly higher in SSNS than in SRNS. The enhanced NPNT gene expression with subsequent elevated serum level during recovery in SSNS patients indicates its role in nephron regeneration. On the other hand, in SRNS, the expression of NPNT was not enhanced by corticosteroid treatment. The lower serum levels of NPNT in SRNS than controls may be attributed to its loss in urine with the massive proteinuria. These findings may suggest NPNT as a possible differentiating biomarker between SSNS from SRNS.

To our knowledge this is the first study to assess NPNT as a biomarker to differentiate SSNS from SRNS, however, many studies had been performed investigating its role in renal development and regeneration after kidney injuries [12, 16, 17].

Enhanced NPNT expression in SSNS patients may agree with Müller-Deile et al., 2017, who showed that the reduced

glomerular levels of NPNT is a novel probable mechanism for proteinuria development in active glomerular diseases [18].

Also, our findings are in agreement with Kashani et al.; 2015, who reported NPNT as one of the novel biomarkers that indicate kidney repair after acute kidney injury; however, they recommended further investigations to verify its performance in the clinical setting [19].

Moreover, Cheng et al., 2008 declared enhanced expression of NPNT in regenerating nephrons in an experimental model during recovery from acute kidney injury [17].

Linton et al., 2007 reported that nephronectin is essential for kidney development through integrin α8β1-mediated stimulation of glial cell line-derived neurotrophic factor (GDNF) expression. And, they also showed that the lack of functional NPNT in embryos is associated with kidney agenesis or hypoplasia [12].

Nakatani et al., 2012 studied Nephronectin expression in glomeruli from various kidney diseases. They reported its lower expression in almost all active glomerular diseases: lupus nephritis, membranous glomerulonephritis, IgA glomerulonephritis, proliferative glomerulonephritis, crescentic glomerulonephritis, amyloidosis, light chain deposition disease, and many other kidney diseases, such as segmental glomerulosclerosis and hypertensive nephropathy [20].

Soluble urokinase plasminogen activator receptor (suPAR) has been the most extensively studied biomarker of SRNS. However, contradictory results had been declared. In 2015, Peng Z et al., showed that serum suPAR may help to differentiate steroid resistance from steroid-sensitive NS in children with high specificity but low sensitivity [21].

NPNT is the preferred α8β1 integrin ligand and mice models deficient in NPNT showed similar phenotype to those with α8β1 integrin deficiency [12]. uPAR acts through the activation of podocyte β3-integrin signaling. β3-integrin activation is associated loss of podocyte α3 and α5 integrins with subsequent podocyte effacement and detachment from the GBM [22]. Because α3 and β1 are the most important integrins involved in podocytes effacement, likely, combined NPNT and suPAR estimation may give promising results.

Although FSGS is the most common pathological condition causing SRNS, the underling pathology of SRNS includes a variety of other disease conditions. Since 2013, many studies had suggested uPAR as marker of FSGS. However, their findings have been extremely controversial [23–31].

Wei et al., showed elevated suPAR concentrations in primary FSGS compared to healthy controls and to patients with MCD in both children and adults and defined a threshold of 3000 pg/ml might distinguish FSGS from other glomerulopathies [26]. Also, Li et al. found uPAR to be specifically elevated in some patients with FSGS compared to MCD and MN and suggested that suPAR assay may help to predict steroid response in patients with primary FSGS [27].

However, Schlöndorff showed that serum suPAR can not reliably distinguish FSGS from other proteinuric glomerular diseases and denied the validity of serum suPAR as a biomarker of primary or secondary FSGS [28]. Moreover, Sinha et al., Maas et al., Meijers et al., and Bock et al. also found that suPAR do not distinguish FSGS from other causes of NS [22, 29–31].

The present study demonstrated, for the first time, that Nephronectin may be a candidate biomarker differentiating SSNS from SRNS with promising sensitivity and specificity. However, due to the relative small sample size used in this study; further studies with larger number of samples are recommended.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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