Table 3 continued

Non–IgAN cases				
	β	Coefficient	95 % CI	*p*
BMI	0.036	0.161	–0.005 to 0.077	0.086
S–Cr	0.333	0.264	0.100 to 0.567	<0.01
MAP	0.002	0.036	–0.009 to 0.013	0.705
Model 2				
Age	–0.003	–0.070	–0.013 to 0.006	0.485
BSA	0.463	0.096	–0.448 to 1.374	0.316
S–Cr	0.324	0.257	0.083 to 0.565	<0.0 1
MAP	0.004	0.070	–0.007 to 0.015	0.451
Nephrotic cases				
Model 1				
Age	–0.125	–0.563	–0.349 to 0.099	0.244
BMI	–0.414	–0.606	–1.160 to 0.332	0.244
S–Cr	0.033	0.016	–1.764 to 1.831	0.968
MAP	–0.028	–0.144	–0.200 to 0.144	0.727
Model 2				
Age	–0.099	–0.447	–0.355 to 0.157	0.409
BSA	–4.691	–0.450	–19.248 to 9.865	0.489
S–Cr	–0.026	–0.012	–2.418 to 2.366	0.981
MAP	0.030	–0.156	–0.251 to 0.191	0.769

Model 1: Independent variables are Age, BMI, S-Cr, MAP. Model 2: Independent variables are Age, BSA, S-Cr, MAP

BMI body mass index, *BSA* body surface area, *S–Cr.* serum creatinine, *MAP* mean arterial pressure

It has been reported that the amount of urine protein is affected by obesity [2]. In general, the physical constitution of Japanese people is smaller compared with that of people in the West. The Western definition of obesity is usually BMI of 30 kg/m^2, while the Japanese definition is 25 kg/m^2, as determined by Japan Society for the Study of Obesity (JASSO) [18]. Glomerular filtration rate (GFR) is usually adjusted by BSA at a value of 1.73 m^2 worldwide, because 1.73 m^2 is the average BSA in the general adult population in Western countries. However, the mean BSA of Japanese people seems to be smaller than 1.73 m^2. In chemotherapy for malignancy, anti-cancer agents are usually adjusted by BSA. In the relevant literature, 1.75 m^2 is seen to have been determined as a larger BSA size [19, 20]. Therefore, we set the parameters between larger and smaller physical constitutions as BMI 25 kg/m^2 and BSA 1.73 m^2, respectively.

Both of these indexes of Japanese physical constitutions, BMI $\geq$25 kg/m^2 and BSA $\geq$1.73 m^2, affected the AUPE in the evaluation of all the study patients. The higher BMI and BSA groups had significantly larger AUPEs ($p < 0.001$; $p < 0.001$) (Fig. 1). Interestingly, in the evaluation of each histological group, the FSGS and non-IgA groups did not show any significant differences between larger and

Table 4 Multiple linear regression analysis for the amount of urine protein

MCNS cases				
	β	coefficient	95 % CI	p
All cases				
Model 1				
Age	–0.038	–0.152	–0.063 to –0.12	<0.01
BMI	0.184	0.162	0.063 to 0.306	<0.01
S–Cr	0.395	0.067	–0.219 to 1.009	0.207
MAP	0.027	0.068	–0.016 to 0.070	0.217
Model 2				
Age	–0.018	–0.073	–0.045 to 0.009	0.194
BSA	5.535	0.221	2.775 to 8.295	<0.01
S–Cr	0.212	0.036	–0.409 to 0.834	0.502
MAP	0.023	0.057	–0.020 to 0.065	0.292
Non–nephrotic cases				
Model 1				
Age	0.013	0.206	–0.002 to 0.027	0.09
BMI	0.014	0.040	–0.064 to 0.092	0.723
S–Cr	–0.102	–0.096	–0.350 to 0.146	0.415
MAP	0.016	0.173	–0.005 to 0.037	0.138
Model 2				
Age	0.014	0.231	–0.001 to 0.029	0.061
BSA	0.745	0.106	–0.803 to 2.293	0.341
S–Cr	–0.112	–0.106	–0.359 to 0.134	0.368
MAP	0.015	0.161	–0.006 to 0.036	0.164
Nephrotic cases				
Model 1				
Age	–0.056	–0.253	–0.081 to 0.031	<0.01
BMI	0.087	0.088	–0.031 to 0.204	0.148
S–Cr	0.976	0.160	0.264 to 1.688	<0.05
MAP	0.010	0.028	–0.034 to 0.054	0.654
Model 2				
Age	–0.042	–0.188	–0.069 to 0.014	<0.01
BSA	3.705	0.170	0.917 to 6.494	<0.01
S–Cr	0.775	0.127	0.046 to 1.503	<0.05
MAP	0.003	0.009	–0.040 to 0.047	0.875

MN cases				
	β	Coefficient	95 % CI	*p*
ALL cases				
Model 1				
Age	0.012	0.037	–0.014 to 0.038	0.373
BMI	0.187	0.183	0.103 to 0.271	<0.01
S–Cr	1.608	0.216	1.000 to 2.215	<0.01
MAP	0.010	0.038	–0.011 to 0.030	0.366
Model 2				
Age	0.024	0.075	–0.004 to 0.051	0.089
BSA	2.760	0.143	1.086 to 4.435	<0.01
S–Cr	1.555	0.209	0.938 to 2.172	<0.01

Table 4 continued

MN cases				
	β	Coefficient	95 % CI	*p*
MAP	0.012	0.047	−0.009 to 0.033	0.262
Non-nephrotic cases				
Model 1				
Age	0.021	0.237	0.010 to 0.032	<0.01
BMI	0.009	0.030	−0.027 to 0.044	0.622
S-Cr	0.043	0.014	−0.331 to 0.416	0.822
MAP	0.009	0.117	<0.001 to 0.018	0.054
Model 2				
Age	0.018	0.204	0.006 to 0.031	<0.01
BSA	−0.335	−0.061	−1.074 to 0.405	0.373
S-Cr	0.112	0.037	−0.282 to 0.505	0.576
MAP	0.010	0.130	0.001 to 0.019	<0.05
Nephrotic cases				
Model 1				
Age	−0.018	−0.058	−0.054 to 0.018	0.333
BMI	0.184	0.186	0.067 to 0.301	<0.01
S-Cr	0.787	0.128	0.067 to 1.507	<0.05
MAP	−0.008	−0.035	−0.037 to 0.020	0.563
Model 2				
Age	0.000	<0.001	−0.038 to 0.038	1.00
BSA	3.374	0.181	1.088 to 5.660	<0.01
S-Cr	0.777	0.126	0.055 to 1.498	<0.05
MAP	−0.070	−0.020	−0.036 to 0.021	0.619
IgAN cases				
	β	Coefficient	95 % CI	*p*
ALL cases				
Model 1				
Age	0.004	0.041	0.000–0.008	0.079
BMI	0.031	0.086	0.015–0.047	<0.01
S-Cr	0.384	0.215	0.304–0.464	<0.01
MAP	0.018	0.176	0.013–0.023	<0.01
Model 2				
Age	0.005	0.054	0.001–0.009	<0.05
BSA	0.323	0.045	0.009–0.637	<0.01
S-Cr	0.377	0.211	0.296–0.457	<0.01
MAP	0.019	0.187	0.014–0.024	<0.05
Non-nephrotic cases				
Model 1				
Age	0.001	0.025	−0.001 to 0.004	0.299
BMI	0.013	0.063	0.004 to 0.023	<0.01
S-Cr	0.251	0.228	0.201 to 0.301	<0.01
MAP	0.013	0.218	0.010 to 0.016	<0.01
Model 2				
Age	0.002	0.034	−0.001 to 0.004	0.151
BSA	0.250	0.226	0.199 to 0.300	<0.01

Table 4 continued

IgAN cases				
	β	Coefficient	95 % CI	*p*
S-Cr	0.014	0.233	0.011 to 0.016	<0.01
MAP	0.016	0.004	−0.164 to 0.196	0.863
Nephrotic cases				
Model 1				
Age	0.005	0.044	−0.017 to 0.027	0.662
BMI	0.084	0.211	0.005 to 0.163	<0.05
S-Cr	−0.012	−0.008	−0.292 to 0.269	0.935
MAP	−0.007	−0.058	−0.034 to 0.019	0.584
Model 2				
Age	0.009	0.081	−0.013 to 0.031	0.419
BSA	2.781	0.308	1.003 to 4.559	<0.01
S-Cr	−0.056	−0.041	−0.331 to 0.220	0.689
MAP	−0.006	−0.048	−0.032 to 0.020	0.639

Model 1: Independent variables are Age, BMI, S-Cr, MAP. Model 2: Independent variables are Age, BSA, S-Cr, MAP

BMI body mass index, *BSA* body surface area, *S-Cr.* serum creatinine, *MAP* mean arterial pressure

smaller physical constitutions as evaluated by BMI and BSA (Fig. 2). There were no significant differences in the comparison of MPGN between the larger and smaller BSA groups. In MCNS and MN groups, the main histological damage is in the glomerular capillary walls. Conversely, in the remaining groups, the main histological damage is found both on the glomerular capillary walls and in the mesangial areas. We suspected that this histological difference might produce the distinguished results in the types of glomerular diseases.

We performed detailed examinations to divide the patients into two groups, the nephrotic and non-nephrotic groups, because the nephrotic patients of the larger physical constitution groups may have two conditions: systemic edema and obesity [20]. Patients with MCNS and MN showed significant differences, but even in the evaluation of nephrotic cases, patients with FSGS and non-IgA did not show any significant differences in the AUPE between the larger and smaller BMI or BSA groups.

This result may indicate that the capillary wall damage seen in patients with MCNS and MN is easily affected by hyperfiltration associated with obesity or larger physical constitution. The condition of overfilling, dependent on sodium retention in nephrotic syndrome, may worsen the permeability of the glomerular capillary walls in MCNS and MN [21]. Recently, sodium retention derived from activation of the amiloride-sensitive epithelial sodium channel (ENaC) has attracted attention in nephrotic syndrome [22]. The larger physical condition including systemic edema and obesity might more strongly affect the

AUPE in the condition of increased capillary wall permeability of nephrotic MCNS and MN cases. Conversely, we hypothesized that the complex damage to the glomerular capillary walls and mesangial areas seen in FSGS and non-IgA may produce severe proteinuria that is influenced by factors other than glomerular hyperfiltration and/or overfilling condition, even in patients with obesity or larger physical constitution.

To confirm this hypothesis, we performed multivariable analysis. In the analysis in all cases, BMI and BSA were certainly the significant independent factors contributing to the AUPE (Table 3). On the other hand, BMI and BSA were not the significant contributors to the AUPE in the analysis of FSGS and non-IgA. Even in nephrotic cases, BMI and BSA did not significantly affect AUPE in FSGS and non-IgAN. In these groups, MAP and S–Cr were significant independent factors contributing to the AUPE, respectively (Table 3). The main influencing causes of proteinuria were apparently different between the MCNS/MN group and the FSGS/non-IgA group. In the analysis of the non-nephrotic groups (Fig. 4), the effects of larger physical constitutions on the AUPE seemed to disappear in MCNS and MN. This result supports the fact that larger physical constitutions including obesity easily affect the AUPE in particular nephrotic syndrome that have main histological damages on GBM. Both IgAN and non-IgAN are indeed mesangial proliferative GN, while the BMI and BSA significantly affected the AUPE of IgAN in multivariate analysis (Table 4). Unfortunately, we could not understand this conflicting point. IgAN cases were youngest and their mean blood pressure was lowest in our all cases. From this feature, larger physical condition including edema and obesity might easily affect the glomerular capillary permeability in IgAN compared to non-IgAN.

We noticed some different effects between BMI and BSA in MPGN and IgAN in nephrotic cases (Figs. 4, 5). Originally, BMI parallels to body fat mass and BSA to body volume. From the formula, BMI easily increases a numerical value compared to BSA when body weight increases from the edema. Thus BSA might fail to show significant differences in nephrotic cases of MPGN and FSGS. In non-nephrotic cases FSGS and non-IgAN showed the inverse results (Figs. 6, 7). We could not understand the reason of this.

Woo et al. reported that FSGS has dramatically increased in the last decade in Asian countries [4, 23]. These investigators suspected that socioeconomic factors, including the rising obesity rate, might play a significant role in this phenomenon. The etiologies of FSGS are complicated; among them, hypertension has been of concern as another important cause in animal models and human FSGS [23, 24]. In FSGS, hypertension induces the reduction of nephron mass from arteriosclerosis [25] and podocyte stress due to glomerular hypertension [14]. These multifactorial conditions might lead to the increase in urine protein in FSGS.

Limitations

The limitations of this study relate to the melding of systemic edema and obesity, and the unclear definitions of larger physical constitutions. The patients with nephrotic syndrome were thought to have systemic edema and obesity in the larger constitution groups at the same time. Thus, we could not insist that obesity purely affected the AUPE in this study. We used two types of indexes of physical constitution. BMI is generally used as a marker to determine obesity, but it is unclear whether this marker is a precise marker of body volume. We selected another marker, BSA, which is used to constitutionally correct the glomerular filtration rate; therefore, we thought it might be an appropriate index to evaluate the AUPE to some extent. But we could not make a clear decision on an applicable value of BSA to use to indicate the boundary between larger and smaller physical constitutions.

Conclusion

This study confirmed that the larger physical constitutions including obesity, BMI $\geq$25 kg/m^2 and BSA $\geq$1.73 m^2, have a significant impact on the increase in the AUPE in primary glomerulonephritis and especially in nephrotic patients. At the same time, patients with FSGS and non-IgA did not show the same tendency, rather, systemic blood pressure and renal dysfunction were the major causes of proteinuria in the patients with FSGS and non-IgA. Larger physical constitutions including obesity distinctively affect the AUPE in the different types of primary glomerulonephritis.

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Conflict of interest The authors have declared that no Conflict of interest exists.

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ORIGINAL ARTICLE

Clinicopathological characteristics of M-type phospholipase A2 receptor (PLA2R)-related membranous nephropathy in Japanese

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Abstract
Background Recent studies have suggested that assessments of serum antibodies against M-type phospholipase A2 receptor (PLA2R) and the glomerular expression of PLA2R antigen in biopsy specimens are useful for the diagnosis of primary membranous nephropathy (MN). In this study, we assessed both of them and investigated the clinicopathological characteristics of PLA2R-related Japanese MN.
Methods We retrospectively enrolled 22 primary and 3 secondary Japanese patients whose serum samples and renal specimens were collected before treatment. According to the findings of serum antibodies and antigen in glomeruli, the primary MN patients were classified into PLA2R-related or -unrelated MN. We compared their clinicopathological findings, including IgG subclass staining, and electron microscopic findings, and evaluated the predictors of proteinuria remission.
Results In primary MN, 16 patients (73 %) were classified into the PLA2R-related group, and 6 patients into the PLA2R-unrelated group. There was no significant difference in baseline laboratory data and electron microscopic findings, except for eGFR and serum IgG levels. IgG4-dominant deposition was more common in the related group (63 vs. 0 %). The 10 PLA2R-related patients with dominant IgG4 deposition had a lower rate and prolonged time in remission compared with the 6 PLA2R-related patients with non-dominant IgG4 (log-rank, $p = 0.032$). Furthermore, dominant IgG4 deposition was an unfavorable predictor of remission by multivariable Cox proportional hazard analysis.
Conclusions Assessments of both serum PLA2R antibodies and PLA2R antigen in glomeruli were more sensitive for the diagnosis of PLA2R-related MN, and among affected Japanese patients, those with dominant IgG4 deposition had worse clinical outcomes.

Keywords Electron microscopic findings · IgG subclass staining · Membranous nephropathy · Phospholipase A2 receptor · Western blotting

Introduction

Membranous nephropathy (MN) is the most common cause of nephrotic syndrome in adults. MN is characterized by immune complex deposition in the subepithelial space of glomerular capillaries. In 10–20 % of patients, MN is associated with an underlying disease, such as autoimmune disease (e.g., systemic lupus erythematosus), infection, drugs and malignancies [1]. When no underlying cause is identified, the disease is classified as primary (idiopathic). It has long been suspected that primary MN is evoked by the in situ formation of immune complexes as circulating antibodies react with a podocyte antigen. In 2009, M-type phospholipase A2 receptor (PLA2R) was identified as a target podocyte antigen in primary MN. Circulating

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autoantibodies against PLA2R were found in 70 % of patients with idiopathic MN, but not in those with secondary MN or other glomerular diseases [2]. The level of circulating anti-PLA2R autoantibody correlated with clinical disease activity and was useful to monitor response to treatment [3–5]. In addition, assessment of the glomerular expression of PLA2R antigen in biopsy specimens was also useful for the diagnosis of primary MN [6, 7]. Svobodova et al. [7] showed that assessment of both circulating anti-PLA2R antibodies and PLA2R antigen in biopsy might better categorize patients into different groups than only assessing anti-PLA2R antibodies, even during active disease.

In this study, we assessed both serum anti-PLA2R antibodies and PLA2R antigen in glomeruli at the same time before treatment and defined the patients with either elevated anti-PLA2R antibody in the serum or enhanced PLA2R in glomeruli as having PLA2R-related MN. We assessed their clinicopathological characteristics and clinical outcomes in comparison with those in PLA2R-unrelated primary MN and secondary MN.

Materials and methods

Patients

We retrospectively enrolled 25 Japanese MN patients who were admitted to Kanazawa Medical University Hospital between 1998 and 2013, and whose serum samples were collected before treatment at the time of renal biopsy. We followed these patients for at least 6 months (median (IQR), 26 [16.8–35.3] months). Diagnosis was confirmed in all patients by percutaneous needle renal biopsy. To detect secondary causes of MN, clinical workup including detailed medical history and physical examination, serological analysis (tumor markers, lupus autoantibodies, hepatitis B and C) and CT scan were conducted. The patients with lupus nephritis were excluded from this study.

In primary MN, we defined the patients who were either serum anti-PLA2R autoantibody- or glomerular PLA2R-positive as the PLA2R-related group and those who were negative in both serum and glomeruli as the PLA2R-unrelated group. Between these two groups, we compared the baseline clinicolaboratory data, pathological characteristics and clinical outcomes, such as remission rate.

We measured urine protein:creatinine ratio (uPCR) and evaluated clinical status according to Japanese clinical categories as follows: the nephrotic state, that is, the presence of heavy proteinuria (greater than 3.5 g/gCre) and hypoalbuminemia (less than 3.0 g/dL); incomplete remission type II, that is, mean daily proteinuria of 1.0–3.5 g/gCre accompanied by an improvement of serum albumin levels (more than 3.0 g/dL); incomplete remission type I, that is, mean daily proteinuria of 0.3–1.0 g/gCre with normal serum albumin levels (more than 3.0 g/dL); and complete remission, that is, daily proteinuria of less than 0.3 g/gCre with normal serum albumin levels. For the purpose of analysis, we defined remission as improvement of clinical status to the point of incomplete remission type I, according to the criterion of good long-term renal outcome in Japan [8]. The patients were treated non-randomly, depending on the judgment of the doctor in charge of each case. The protocol of this study was approved by the Clinical Study Ethics Review Board of Kanazawa Medical University. Prior to the study, verbal/written informed consent was obtained from all patients (Clinical Study Ethics Review Board of Kanazawa Medical University, Approval No. 80). This study was conducted according to the principles of the Declaration of Helsinki.

Western blotting

The anti-PLA2R autoantibodies in the serum were detected by Western blot analysis with cell lysate of HEK293 cells expressing recombinant PLA2R proteins. HEK293 cells were transfected with coding cDNA of the extracellular domain of PLA2R and homogenized in RIPA buffer (Wako Pure Chemicals, Japan) with protease inhibitor cocktail (Roche Applied Science, USA). Then, the cell lysate was mixed with 4× LDS sample buffer (Invitrogen, USA) and heated for 10 min at 70 °C. Equal amounts of the cell lysate were electrophoresed in 4–12 % Bis–Tris polyacrylamide gel (Invitrogen, USA) under non-reducing conditions and transferred to polyvinylidene difluoride membranes (Invitrogen, USA). The membranes were blocked with Blocking-One (Nacalai Tesque, Japan) and incubated with the serum at a dilution of 1:100. After washing, the membranes were incubated with horseradish peroxidase (HRP)-conjugated mouse anti-human IgG secondary antibody (Abcam) at a dilution of 1:20,000. 5 % Blocking-One in phosphate-buffered saline with 0.05 % Tween 20 (PBST) was used for all primary and secondary antibody dilutions. The immunoreactive bands were visualized by a chemiluminescent technique using ImmunoStar LD (Wako Pure Chemicals, Japan) as an HRP substrate.

Immunohistological staining

Fresh tissue specimens, embedded in OCT compound and frozen in acetone-dry ice mixture, were cut at a thickness of 3 μm on a cryostat. The frozen sections were fixed in 1:1 acetone:methanol and blocked with 10 % goat serum in 0.01 mol/L phosphate-buffered saline (PBS). To detect PLA2R, we used rabbit anti-PLA2R antibodies (Atlas Antibodies) at a dilution of 1:400, followed by fluorescein

isothiocyanate (FITC)-labeled goat anti-rabbit IgG antibodies (Cosmo Bio) at a dilution of 1:640. We detected the IgG subclass in the MN immune deposits with mouse anti-IgG1, -IgG2, -IgG3 and -IgG4 monoclonal antibodies (AbD Serotec), each at a dilution of 1:50, followed by FITC-labeled goat anti-mouse IgG antibodies (Cosmo Bio) at a dilution of 1:160. Immunofluorescence staining intensity was arbitrarily graded on a scale from 0 to 3 (0 negative, 1 weak staining, 2 moderate staining, 3 strong staining) and the median score was calculated (Supplementary Figures S1 and S2).

Electron microscopic examination

The specimens obtained from 24 patients were fixed with glutaraldehyde and osmium tetroxide, embedded in Epon 812, cut into 0.1 μm sections, double-stained with uranyl acetate and lead citrate, and examined with a Hitachi H-7650 electron microscope. For the electron microscopic study, we examined the specimens with emphasis on the phase and synchronicity of subepithelial and intramembranous electron-dense deposits and divided them into two subtypes, namely, homogeneous type with monophasic deposits and heterogeneous type with polyphasic deposits, as previously reported [9, 10]. We also used the Ehrenreich and Churg classification for the ultrastructural staging of MN [11].

Statistical analysis

Continuous measures and ranking scales were summarized using medians (25–75 % interquartile range), whereas categorical measures were summarized using counts and percentages. Mann–Whitney U test was used to assess the differences between two groups. Fisher's exact test was utilized to compare proportions. The Kaplan–Meier life-table method and Cox proportional hazard analysis were performed to evaluate predictors of remission of proteinuria. In addition, Cox proportional hazard models were developed with relevance to remission with subsequent addition of sociodemographic (age and sex), clinical variables (eGFR and uPCR), pathological findings (PLA2R, IgG subclass staining and electron microscopic stage), and treatment. P values of less than 0.05 were considered statistically significant. The Cox proportional hazard analysis was conducted using Stat Flex, version 6.0 (Artech, Japan), and the others were conducted using SPSS, version 20.0 (Chicago, IL, USA).

Results

PLA2R of primary and secondary MN in Japanese

In this study, 22 primary MN patients and 3 secondary MN patients were enrolled. The causes of secondary MN were malignancy (malignant thymoma, $n = 1$), bucillamine ($n = 1$) and chronic graft versus host disease (GVHD) after peripheral blood stem cell transplantation ($n = 1$).

In secondary MN, no patient had autoantibody in serum and glomerular PLA2R deposits. On the other hand, in 12 primary MN patients (55 %), circulating PLA2R autoantibodies were positive with glomerular PLA2R in 10 patients, but not in 2 patients. Of the 10 primary MN patients with no detectable PLA2R autoantibodies in serum, 4 patients had glomerular PLA2R deposits. According to these findings, 16 patients (73 %) were classified into the PLA2R-related group and 6 patients into the PLA2R-unrelated group.

Clinicopathological characteristics of primary MN related to PLA2R positivity

The clinicolaboratory data and pathological characteristics at baseline are presented in Table 1. There was no significant difference in clinicolaboratory data, except for eGFR and serum IgG levels. The intensity of IgG4 deposits was much higher in the PLA2R-related group (3.0 vs. 0.5, $P = 0.010$). Then, IgG4 pre- or co-dominant patients were more common in the PLA2R-related group (63 vs. 0 %, $P = 0.0152$). There was no significant difference in the electron microscopic findings.

Clinical outcomes of primary MN

The primary MN patients were treated using supportive therapy ($n = 11$, 50 %), corticosteroid alone ($n = 2$, 9.1 %), cyclosporine with steroid ($n = 6$, 27.3 %) or mizoribine with steroid ($n = 3$, 13.6 %). During the follow-up period, no patient developed end-stage renal failure (ESRF). The results of the univariate time-dependent analysis by the Kaplan–Meier method are shown in Fig. 1. Although there was no significant difference in the remission rate and the time to remission between the PLA2R-related and PLA2R-unrelated groups (Fig. 1a), the 10 PLA2R-related patients with dominant IgG4 deposition had a low rate and prolonged time in remission compared with the 6 PLA2R-related patients with non-dominant IgG4 (log-rank, $p = 0.032$, Fig. 1b). The clinical predictors of remission in the multivariable Cox proportional hazard analysis are presented in Table 2. It is revealed that dominant IgG4 deposition was an unfavorable predictor and immunosuppressive therapy was a favorable predictor of remission.

In patients whose serum antibodies were positive at baseline, with the exception of one patient, we assessed this variable again at the end of the follow-up period (Table 3 and Supplementary Figure S3). In 4 of 8 patients (50 %) who achieved remission, the serum antibodies became

Table 1 Clinicolaboratory data and pathological characteristics of primary MN at baseline

	PLA2R-related [$n = 16$]	PLA2R-unrelated [$n = 6$]	P value
Age (years)	64.5 [61–70]	70 [64–74]	0.336
Male/female	12/4	4/2	0.541
Follow-up (months)	26 [13–36]	24 [16–34]	0.914
Serum creatinine (mg/dl)	0.77 [0.65–0.89]	0.96 [0.80–1.41]	0.083
eGFR (ml/min/1.73 m^2)	75.2 [64.7–82.3]	59.3 [28.9–68.6]	0.049*
Serum albumin (g/dl)	2.35 [2.05–3.05]	2.70 [1.90–3.40]	0.684
Serum IgG (mg/dl)	691.5 [517.5–989.5]	1,045.0 [1,038.0–1,079.0]	0.018*
uPCR (g/gCre)	10.0 [5.3–12.8]	5.5 [3.7–8.1]	0.261
Immunohistological staining			
IgG1 score	1.5 [0.0–2.5]	1.5 [1.0–2.0]	0.909
IgG2 score	0.0 [0.0–1.0]	0.0 [0.0–0.0]	0.089
IgG3 score	1.0 [0.0–1.5]	0.0 [0.0–1.0]	0.448
IgG4 score	3.0 [1.0–3.0]	0.5 [0.0–1.0]	0.010*
IgA score	0.0 [0.0–1.0]	0.0 [0.0–1.0]	0.763
C1q score	0.0 [0.0–1.0]	0.5 [0.0–1.0]	0.738
C3 score	3.0 [0.5–3.0]	1.0 [0.0–2.0]	0.143
Electron microscopic findings	PLA2R-related [$n = 15$]	PLA2R-unrelated [$n = 6$]	P value
Ehrenreich–Churg stage	2.0 [1.0–2.5]	1.5 [1.0–2.0]	0.480
Subtype homogeneous	14 [93 %]	5 [83 %]	0.500
Subtype heterogeneous	1 [7 %]	1 [17 %]	

PLA2R M-type phospholipase A2 receptor, *MN* membranous nephropathy, *uPCR* urine protein/creatinine ratio

* P values of less than 0.05

negative. On the other hand, in all patients who did not achieve remission, the serum antibodies were still positive.

Discussion

Although the exact mechanisms and the role of PLA2R in the pathogenesis of primary MN are currently unknown, PLA2R is probably one of the intrinsic glomerular antigens. In this study, we collected serum samples before treatment at the time of renal biopsy, and assessed both serum anti-PLA2R antibodies and glomerular PLA2R deposits. In previous studies, the prevalence of serum anti-PLA2R antibodies by western blotting in patients with primary MN ranged from 69 to 82 % [2, 4, 5, 12, 13] and the prevalence of glomerular PLA2R deposits ranged from 69 to 84 % [6, 7, 14, 15]. In this study, we detected serum anti-PLA2R antibodies in 55 % and glomerular PLA2R deposits in 64 % of patients with primary MN. The reason for the low prevalence in our serum antibody detection is unclear at this time. A larger Japanese study ($n = 96$) by our colleagues showed similar results (50 %, unpublished data). In other Asian countries, on the other hand, the reported prevalence of serum anti-PLA2R antibodies was 82 % in a Chinese study [12] and 69 % in a Korean study [13]. In this study, combined with the results of glomerular PLA2R deposits, the sensitivity of the diagnosis of PLA2R-related MN rose to 73 %.

In previous studies, serum anti-PLA2R antibodies and PLA2R antigen in glomeruli were largely negative in patients with secondary causes of MN including lupus nephritis, HBV, and malignancy-associated MN [12, 15]. We also examined 3 patients with representative cause of secondary MN (1 malignancy, 1 GVHD, and 1 bucillamin) and found that no patient had autoantibody in serum and glomerular PLA2R deposits.

Similar to previous studies [7, 14], we found that 4 patients had no circulating antibodies, although they had PLA2R detected in glomeruli, while 2 patients with circulating antibodies had no detectable PLA2R in glomeruli. The findings of serum negativity and glomerular positivity were explained by the rapid clearance of antibodies from the blood or by the late sampling of patients when proteinuria persisted because of irreversible ultrastructural changes [7, 14]. However, there was no significant difference in proteinuria at baseline and time to remission between the patients with serum negativity and glomerular positivity and the patients with both serum and glomerular positivity in this study. On the other hand, for the patients with circulating antibodies but without detectable PLA2R in glomeruli, it was speculated that the antibodies were not nephritogenic or that epitopes were poorly accessible at the time of renal biopsy [7, 14]. In this study, circulating antibodies were not detected after remission in 2 patients with serum positivity and glomeruli negativity at baseline (Table 3). Finally, neither PLA2R antigen in glomeruli nor

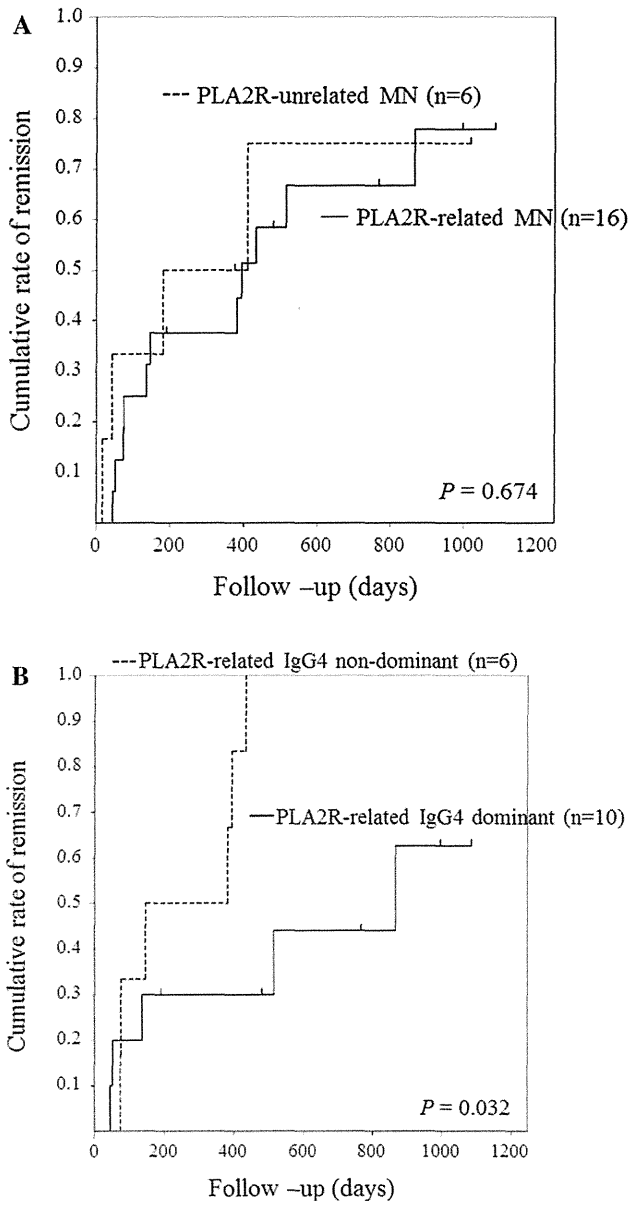

Fig. 1 Kaplan–Meier curves of remission in Japanese membranous nephropathy. **a** There was no significant difference in the remission rate and the time to remission between the PLA2R-related and -unrelated groups. **b** The 10 PLA2R-related patients with dominant IgG4 deposition had a low rate and prolonged time in remission compared with the 6 PLA2R-related patients with non-dominant IgG4 (log-rank, $p = 0.032$)

PLA2R antibody in serum was found in 6 primary MN patients. It is possible that they have an as yet undetected cause of MN or other recently described antigens, such as alpha-enolase, aldose reductase and SOD2 [16].

IgG subtype analysis in glomeruli has been carried out for the discrimination between primary (idiopathic) and secondary MN. IgG4-dominant staining is associated with primary MN, whereas IgG1, IgG2 and IgG3 dominate in the deposits of secondary MN [17–19]. Serum autoantibodies to PLA2R are largely IgG4, and IgG4 could be co-localized with the PLA2R antigen within the glomerular immune deposits of idiopathic MN [2, 3], except for some cases such as monoclonal IgG3-kappa targeting the PLA2R [20]. Consistent with these findings, in our PLA2R-related group, IgG4-dominant deposits in glomeruli were more common than in the PLA2R-unrelated group and secondary MN patients.

Furthermore, IgG4 dominancy was an unfavorable predictor of remission. Hofstra et al. [3] found that spontaneous remissions were less likely to occur in patients with high serum IgG4 antibody titers to PLA2R. Although IgG4 does not activate the classic complement pathway, there are usually abundant deposits of complement, including C4 and C3, in the immune deposits in primary MN. A recent preliminary study reported that degalactosylated anti-PLA2R IgG4 can bind mannan-binding lectin, which suggests the possibility that the lectin pathway of complement might be activated in glomerular IgG4-predominant immune deposits [21]. We speculated that two characteristics in the immune regulation of IgG4 were relevant to the worse remission of proteinuria. One is the finding that the production of IgG4 antibodies seems to be driven by T helper 2 (Th2) cytokine activation in primary MN [22]. The other is its tendency to appear only after prolonged immunization. It usually takes many months of repeated antigen exposure before IgG4 responses become prominent [23]. In this regard, Huang et al. [24] found, in early stages of MN, that IgG1 was the dominant IgG subclass, whereas IgG4 became dominant in later stages. We also found that almost all IgG4-dominant patients (8 of 10) had other IgG subclass deposits to some extent.

Then, we hypothesized that cases of PLA2R-related MN, if caused by immune reactions evoked by repeated exposure to the intrinsic antigen, would have later stages or the heterogeneous type with a different phase of electron-dense deposits. However, there was no significant difference in electron microscopic findings between the PLA2R-related and -unrelated groups. Moreover, early electron-dense deposits such as stage 1 including IgG4 were also observed in the IgG4-predominant or co-dominant group. These findings may suggest that co-localized non-complement-activating IgG4 antibodies and other IgG subclasses such as IgG1, 2 and 3 can augment complement-activating injury, as previously reported in a mouse antibody-mediated allograft rejection model [25].

We found that four patients whose serum antibodies were still positive even after they achieved remission. Among serum positive and negative patients after remission, there was no significant difference about treatment and IgG subclass staining. In previous studies, although there were few atypical patients [4, 7], it was found that anti-PLA2R antibodies disappeared in advance of

Table 2 The multivariate cox proportional hazard model for remission ($n = 22$)

Predictors	Model 1[a]			Model 2[b]		
	HR	95 % CI	*P* value	HR	95 % CI	*P* value
Age (per 5-year interval)	1.35	0.76–2.40	0.299	1.64	0.64–4.17	0.299
Female (vs. male)	2.76	0.75–10.1	0.125	6.02	0.49–73.9	0.160
eGFR (per 10 ml/min/1.73 m^2)	1.56	0.96–2.54	0.071	2.01	0.82–4.91	0.124
uPCR (per 1.0 g/gCre)	–	–	–	0.95	0.72–1.27	0.771
PLA2R-related (vs. -unrelated)	1.24	0.29–5.20	0.759	1.41	0.30–6.55	0.659
IgG4-dominant (vs. non-dominant)	0.08	0.01–0.51	0.007*	0.02	0.00–0.91	0.044*
Ehrenreich–Churg stage	–	–	–	0.74	0.21–2.60	0.643
Immunosuppressive therapy (vs. supportive therapy)	3.39	1.20–47.6	0.140	7.58	1.20–47.6	0.030*

HR hazard ratio, *CI* confidence interval, *PLA2R* M-type phospholipase A2 receptor, *uPCR* urine protein/creatinine ratio

[a] Model 1: adjusted for age, sex, eGFR, relevance to PLA2R, IgG4 dominancy, and treatment

[b] Model 2: model 1 with additional adjustment for uPCR and Ehrenreich–Churg stage

* *P* values of less than 0.05

Table 3 Change of serum anti-PLA2R antibody positivity in primary MN patients whose serum antibodies were positive at baseline ($n = 11$)

Patient	Baseline				Treatment	Remission	Follow-up	
	uPCR (g/gCre)	Serum PLA2R	Biopsy PLA2R	IgG4 dominancy			uPCR (g/gCre)	Serum PLA2R
A	3.21	(+)	(+)	Dominant	Supportive	Yes	0.16	(+)
B	19.31	(+)	(+)	Dominant	IS	Yes	0.05	(+)
C	5.82	(+)	(+)	Dominant	Supportive	Yes	0.06	(−)
D	4.7	(+)	(+)	Non-dominant	Supportive	Yes	0.45	(+)
E	9.88	(+)	(+)	Dominant	IS	Yes	0.18	(+)
F	2.15	(+)	(+)	Non-dominant	IS	Yes	0.25	(−)
G	7.82	(+)	(+)	Dominant	IS	No	10.12	(+)
H	12.38	(+)	(+)	Dominant	Supportive	No	8.07	(+)
I	6.84	(+)	(+)	Dominant	Supportive	No	5.79	(+)
J	21.34	(+)	(−)	Dominant	IS	Yes	0.15	(−)
K	10.19	(+)	(−)	Non-dominant	IS	Yes	0.03	(−)

PLA2R M-type phospholipase A2 receptor, *uPCR* urine protein/creatinine ratio, *IS* immunosuppressive therapy

remission of proteinuria [4, 5]. Furthermore, anti-PLA2R antibodies appeared prior to recurrence of MN in a transplant recipient [20]. In this study, the findings that serum antibodies still positive after remission of proteinuria might predict recurrence of proteinuria in the near future, or suggest that their antibodies lost nephritogenicity. For instance, it is possible that the antibodies changed their affinity or avidity, their subclass switched to non-complement-activating subclass or, in IgG4 antibodies, their degalactosylation was modified.

Finally, having PLA2R-related MN was not a predictor for remission of proteinuria in this study. However, Hoxha et al. [26] found that high anti-PLA2R antibody titer measured by ELISA was an independent risk factor for not achieving remission. For the purpose of evaluating the clinical outcome, such as remission of proteinuria, quantitative analysis by ELISA might be better than qualitative analysis by western blotting or assessing PLA2R in glomeruli.

In conclusion, assessments of both serum anti-PLA2R antibody and PLA2R antigen in glomeruli at the same time are a more sensitive method for the diagnosis of PLA2R-related primary MN at present. However, there are discrepancies between the presence of serum antibody and the detection of glomerular antigen. In further investigation, on a larger sample of patients, we need to classify PLA2R-related MN patients into three groups according to serum antibody positivity/negativity and glomerular antigen positivity/negativity, and compare their clinicopathological characteristics and clinical outcomes. As expected, IgG4 antibody is an important clue to elucidate the pathogenesis of PLA2R-related MN. Further investigations are necessary to resolve the issue of serum antibodies positivity after remission.

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Conflict of interest None of the authors has any competing interests.

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ORIGINAL ARTICLE

Clinical significance of serum and urinary soluble urokinase receptor (suPAR) in primary nephrotic syndrome and MPO-ANCA-associated glomerulonephritis in Japanese

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Abstract
Background The soluble urokinase receptor (suPAR) has been implicated as a cause of primary focal segmental glomerulosclerosis (FSGS). However, the clinical significance of suPAR in glomerular diseases currently remains unclear.
Methods In this retrospective single-center cohort study, we investigated serum (s-) and urinary (u-) suPAR in patients with primary nephrotic syndrome (NS) (serum/urine: 37/32 cases) and MPO-ANCA-associated glomerulonephritis (ANCA-GN) (serum/urine: 13/11 cases).
Results In pretreatment s- and u-suPAR, no significant differences were observed between the primary NS and ANCA-GN groups or among the pathological types of primary NS. An inverse correlation was noted between pretreatment s-suPAR and eGFR in the primary NS and ANCA-GN groups. A positive correlation was noted between pretreatment u-suPAR and proteinuria in the primary NS group. Furthermore, time-course changes in s- and u-suPAR over 2 months after therapy were associated with the therapeutic responsiveness of primary NS, particularly the differentiation of MCNS from FSGS (s-suPAR: AUC-ROC = 0.905, $p = 0.007$; u-suPAR: AUC-ROC = 0.816, $p = 0.048$). In the ANCA-GN group, a positive correlation was found between pretreatment s-suPAR and clinical severity or crescent formation, whereas u-suPAR was not correlated with these parameters.
Conclusion S- and u-suPAR after therapy may serve as clinical markers to judge the treatment response of untreated NS and differentiate MCNS from FSGS, but not in pretreatment patients. S-, but not u-suPAR may predict the severity of and crescent formation in ANCA-GN.

Keywords suPAR · Nephrotic syndrome · MPO-ANCA-associated glomerulonephritis

Introduction

Focal segmental glomerulosclerosis (FSGS) is a typical disease that manifests steroid-resistant, intractable nephrotic syndrome (NS). Reiser's group reported that the serum-soluble urokinase receptor (s-suPAR) bound to and activated β3 integrin on glomerular podocytes and induced proteinuria and FSGS-like lesions [1–3]. They concluded that suPAR was a humoral factor involved in the development of FSGS. In their study, s-suPAR was high in 2/3 of primary and recurrent FSGS patients after kidney transplantation, whereas no elevation in s-suPAR was noted in patients with other glomerular diseases. FSGS-like lesions can be prevented by the removal of circulating s-suPAR with an anti-suPAR antibody treatment, plasmapheresis, a β3 integrin-inhibitory, low-molecular-weight substance, and cycloRGDfv. Huang et al. [4] reported that s-suPAR was significantly higher in patients with crescentic FSGS than in those with non-crescentic FSGS. They also recently demonstrated that urinary suPAR (u-suPAR) was specifically elevated in patients with primary FSGS and was associated with disease severity [5].

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Recent studies did not discriminate between FSGS, minimal change diseases, or steroid-responsive illnesses in pediatric patients with NS and in patients with biopsy-proven idiopathic FSGS or with non-FSGS diseases [6, 7]. Wada et al. [8] demonstrated that s-suPAR was not a useful clinical marker for FSGS patients in Japan. Otherwise, time-course changes of s-suPAR after therapy could not be assessed in this paper, because of a multicenter cross-sectional study. Moreover, they did not study u-suPAR in Japanese.

The aim of our longitudinal study was to clarify the predictability of long-term therapeutic responses by assessing time-course changes in s- and u- suPAR after therapy. We also investigated the usefulness of suPAR in differentiating FSGS from MCNS, and the clinical significance of suPAR in a typical crescentic disease, MPO-ANCA-associated glomerulonephritis (ANCA-GN).

Materials and methods

Patient population

S-suPAR was measured before the administration of treatments in 37 patients diagnosed with primary NS and treated at Kanazawa Medical University (8 FSGS, 12 MCNS, 15 MN, and 2 MPGN patients) and 13 ANCA-GN patients. U-suPAR was also measured in 32 patients with primary NS (8 FSGS, 10 MCNS, 12 MN, and 2 MPGN patients) and 11 ANCA-GN patients. eGFR was calculated using the predictive equation for Japanese people [9], and urinary protein excretion was determined from the urinary protein/Cr ratio. Renal biopsy was performed in all NS patients and 10 of the ANCA-GN patients. S-suPAR after the initiation of immunosuppressive therapy was measurable in 7 FSGS, 9 MCNS, 9 MN, and 2 MPGN patients. U-suPAR after therapy was also measurable in 7 FSGS, 7 MCNS, 6 MN, and 2 MPGN patients. The time-course changes induced by immunosuppressive therapy were investigated in these patients. S- and u-suPAR were also measured in 20 healthy volunteers. The protocol of this study was approved by the Clinical Study Ethics Review Board of Kanazawa Medical University (No. 197). Prior to this study, verbal/written informed consent was obtained from all patients. The present study was conducted according to principles of the Declaration of Helsinki.

Immunosuppressive therapy protocol

Of all primary NS patients, 71 % (including all FSGS and MCNS patients) received the protocol immunosuppressive therapy: methylprednisolone pulse (MPT)/prednisolone (PSL)/cyclosporine (CyA) combination therapy (Table 1), in which MP was administered for 3 days at 500 mg/day, followed by oral PSL and CyA. PSL was initially administered at 20 mg, while CyA was given at 2 mg/kg/day. These doses were adjusted to target CyA levels of 600–800 ng/mL by 2 h after administration.

Definition of the treatment response of NS and clinical severity of ANCA-GN

The responses of NS to the treatment were assessed based on the definition of the Japanese Society of Nephrology [10]: urinary protein less than 0.3 g/day, between 0.3 and below 1.0 g/day, between 1.0 and below 3.5 g/day, and 3.5 g/day or greater were defined as complete remission (CR), incomplete remission (ICR)-I and -II, and ineffective, respectively. When urinary protein was not decreased to below 1 g/day by the various treatments including steroids and immunosuppressors for 6 months, the condition of the patient was defined as intractable NS.

The clinical severity of ANCA-GN was determined based on the definition of the Japanese Society of Nephrology [11].

Measurement of serum and urinary suPAR

suPAR was measured in sera and urine frozen at −80 °C using a commercial ELISA kit, the Quantikine Human suPAR Immunoassay (R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocol.

Statistical analysis

Data of continuous variables are presented as medians with interquartile ranges. Dunn's test (nonparametric) was used for multiple comparisons of pretreatment s-/u-suPAR and clinical parameters. To compare UP before the treatment between the primary NS and ANCA-GN groups, the Mann–Whitney U test was used. Spearman's correlation coefficient test was used to evaluate the relationships between pretreatment s-/u-suPAR and clinical parameters. However, statistical analysis could not be performed about the correlation between pretreatment u-suPAR and CRP, because almost cases showed negative CRP levels. To compare s- and u-suPAR levels (eGFR, CRP, UP) in the primary NS group between before and after 2 months of treatment, the Wilcoxon's signed-rank test was used. Multiple regression analyses were performed to evaluate the relationship between therapeutic responses (i.e., intractable NS or non-intractable NS) and changes in s-suPAR during 2 months after therapy or s-suPAR at 2 months after therapy while controlling for eGFR and CRP. A ROC analysis was used to evaluate the accuracy of differentiation based on s- and u-suPAR. Stat Flex Ver6

Table 1 Demographic/clinical characteristics

	Primary nephrotic syndrome					ANCA-GN	Normal control
	Total $n = 37$	FSGS $n = 8$	MCNS $n = 12$	MN $n = 15$	MPGN $n = 2$	$n = 13$	$n = 20$
Urinary suPAR measurable *n* (%)	32 (86.5)	8 (100.0)	10 (83.3)	12 (80.0)	2 (100.0)	11 (84.6)	20 (100.0)
Baseline characteristics							
Age (years)	60.0 (40.0–68.0)	48.0 (29.0–68.0)	47.0 (33.5–61.0)[a]	66.0 (60.8–71.3)	24, 76	69.0 (62.3–77.0)	29.5 (25.5–34.0)[A, B]
Gender (male %)	59.5	50.0	58.3	73.3	100.0	46.2	75.0
UP (g/gCr)	9.3 (7.2–12.4)[C]	11.5 (9.7–15.9)	11.4 (9.4–14.3)	7.4 (3.5–8.5)[b, c]	5.6, 9.3	1.2 (0.6–2.0)	Not done*
Selectivity index	0.20 (0.13–0.25)	0.19 (0.16–0.22)	0.14 (0.09–0.24)	0.21 (0.20–0.31)	0.18, 0.38	Not done	Not done
eGFR (mL/min/1.73 m^2)	62.5 (30.4–78.6)[D, E]	39.5 (25.9–79.1)	68.2 (50.2–78.2)	62.5 (28.5–77.1)	95.5, 52.1	14.1 (7.0–39.7)	90.7 (82.1–97.8)
sAlb (g/dL)	1.90 (1.30–2.20)	2.10 (1.55–2.15)	1.30 (1.15–1.40)[d]	2.10 (1.83–2.50)	2.60, 1.80	3.10 (2.20–3.25)[F, G]	5.10 (5.00–5.20)
TC (mg/dL)	343.0 (272.0–437.0)[H, I]	362.5 (325.5–417.5)	469.0 (349.0–514.5)[e]	269.0 (257.0–373.0)	285.0, 233.0	167.0 (149.0–213.3)	202.0 (168.0–225.5)
CRP (mg/dL)	0.10 (0.10–0.26)[J, K]	0.10 (0.10–0.22)	0.22 (0.10–0.34)	0.10 (0.10–0.10)	0.10, 0.10	3.55 (0.34–8.33)	0.05 (0.02–0.08)
Treatments *n* (%)							
MPT + PSL + CyA	25 (67.6)	8 (100.0)	11 (91.7)	5 (33.3)	1 (50.0)	0 (0)	
MPT + PSL + MZB	2 (5.4)	0 (0)	0 (0)	1 (6.7)	1 (50.0)	1 (7.7)	
PSL + CyA	1 (2.7)	0 (0)	0 (0)	1 (6.7)	0 (0)	0 (0)	
PSL + MZB	2 (5.4)	0 (0)	0 (0)	2 (13.3)	0 (0)	0 (0)	
PSL alone	1 (2.7)	0 (0)	0 (0)	1 (6.7)	0 (0)	1 (7.7)	
MPT + PSL + IVCY	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (15.4)	
MPT + PSL	1 (2.7)	0 (0)	1 (8.3)	0 (0)	0 (0)	6 (46.1)	
MPT + PSL + POCY	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	2 (15.4)	
PSL + IVCY	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (7.7)	
Others	5 (13.5)	0 (0)	0 (0)	5 (33.3)	0 (0)	0 (0)	
Outcomes at 6 months							
Urinary protein *n* (%)							
CR	24 (64.9)	7 (87.5)	12 (100.0)	4 (26.7)	1 (50.0)	–	
ICR-I	3 (8.1)	0 (0)	0 (0)	3 (20.0)	0 (0)	–	
ICR-II	8 (21.6)	1 (12.5)	0 (0)	6 (40.0)	1 (50.0)	–	
NS	2 (5.4)	0 (0)	0 (0)	2 (13.3)	0 (0)	–	
Renal function *n* (%)							
ESRD(permanent HD)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	3 (23.1)	
Temporary HD	1 (2.7)	0 (0)	1 (8.3)	0 (0)	0 (0)	2 (15.4)	

Table 1 continued

	Primary nephrotic syndrome					ANCA-GN	Normal control
	Total $n = 37$	FSGS $n = 8$	MCNS $n = 12$	MN $n = 15$	MPGN $n = 2$	$n = 13$	$n = 20$
Not required HD	36 (97.3)	8 (100.0)	11 (91.7)	15 (100.0)	2 (100.0)	8 (61.5)	

The data are presented as median (interquartile range). The multiple comparisons for clinical parameters among primary NS (total), ANCA-GN and control or among the pathological types of primary NS (FSGS vs. MCNS vs. MN) were performed by nonparametric test (Dunn test). The comparison for UP between primary NS and ANCA-GN was performed by Mann–Whitney *U* test

FSGS focal segmental glomerulosclerosis, *MCNS* minimal change nephrotic syndrome, *MN* membranous nephropathy, *MPGN* membranoproliferative glomerulonephritis, *ANCA-GN* anti-neutrophil cytoplasmic antibody positive glomerulonephritis, *UP* indicates urinary protein, *Alb* albumin, *TC* total cholesterol, *CRP* C-reactive protein, *MPT* methylprednisolone pulse therapy, *PSL* prednisolone, *CyA* cyclosporine A, *Mz* mizoribine, *CY* cyclophosphamide, *IV* intravenous, *PO* per os, *CR* complete remission, *ICR* incomplete remission, *NS* nephrotic syndrome, *ESRD* end-stage renal disease, *HD* hemodialysis

[A] $p < 0.01$ vs. primary NS (total), [B] $p < 0.01$ vs. ANCA-GN, [C] $p = 0.000001$ vs. ANCA-GN, [D] $p < 0.01$ vs. ANCA-GN, [E] $p < 0.01$ vs. Control, [F] $p < 0.05$ vs. primary NS (total), [G] $p < 0.01$ vs. control, [H] $p < 0.01$ vs. ANCA-GN, [I] $p < 0.01$ vs. control, [J] $p < 0.01$ vs. ANCA-GN, [K] $p < 0.01$ vs. control, [a] $p < 0.05$ vs. MN, [b] $p < 0.05$ vs. FSGS, [c] $p < 0.05$ vs. MCNS, [d] $p < 0.01$ vs. MN, [e] $p < 0.01$ vs. MN

* The urinary proteins of all normal controls were negative in qualitative analysis

(Artech Co., Ltd., Osaka, Japan) was used as the statistical analysis software. A p value of less than 0.05 was regarded as significant.

Results

Clinical characteristics of primary NS and ANCA-GN patients

The clinical characteristics of primary NS and ANCA-GN patients before therapy are shown in Table 1. Primary NS was diagnosed by renal biopsy in all patients. Of the ANCA-GN patients, 10 of those examined by renal biopsy were histologically diagnosed with crescentic glomerulonephritis. eGFR was significantly lower and CRP was higher in the ANCA-GN group than in the primary NS group (eGFR, $p < 0.01$; CRP, $p < 0.01$). Urinary protein (UP) was significantly lower in the ANCA-GN group than in the primary NS group ($p < 0.00001$). No significant differences were observed in eGFR, CRP, or the selectivity index among the primary NS disease types.

Comparison of pretreatment serum and urinary suPAR

S-suPAR before immunosuppressive therapy was significantly higher in the primary NS and ANCA-GN groups than in the control group ($p < 0.01$, Fig. 1a). On the other hand, no significant differences were noted in s-suPAR before immunosuppressive therapy between the primary NS and ANCA-GN groups (Fig. 1a) or among the disease types of primary NS (Fig. 1b).

U-suPAR before immunosuppressive therapy was also significantly higher in the primary NS and ANCA-GN groups than in the control group ($p < 0.01$, Fig. 1c). Similar to the serum results, no significant differences were noted in u-suPAR before immunosuppressive therapy between the primary NS and ANCA-GN groups (Fig. 1c) or among the disease types of primary NS (Fig. 1d).

Relationships between pretreatment serum and urinary suPAR and clinical parameters

Regarding relationships between pretreatment s-suPAR and clinical parameters (primary NS, Fig. 2; ANCA-GN, Fig. 3), a significant inverse correlation with eGFR was noted in the primary NS and ANCA-GN groups (primary NS, $n = 37$, $\rho = -0.677$, $p < 0.001$, Fig. 2b; ANCA-GN, $n = 13$, $\rho = -0.676$, $p = 0.011$, Fig. 3b). A significant positive correlation with CRP was also noted in the ANCA-GN group ($n = 13$, $\rho = 0.702$, $p = 0.008$, Fig. 3c). A significant positive correlation was noted in the primary NS group ($n = 32$, $\rho = 0.576$, $p = 0.001$,

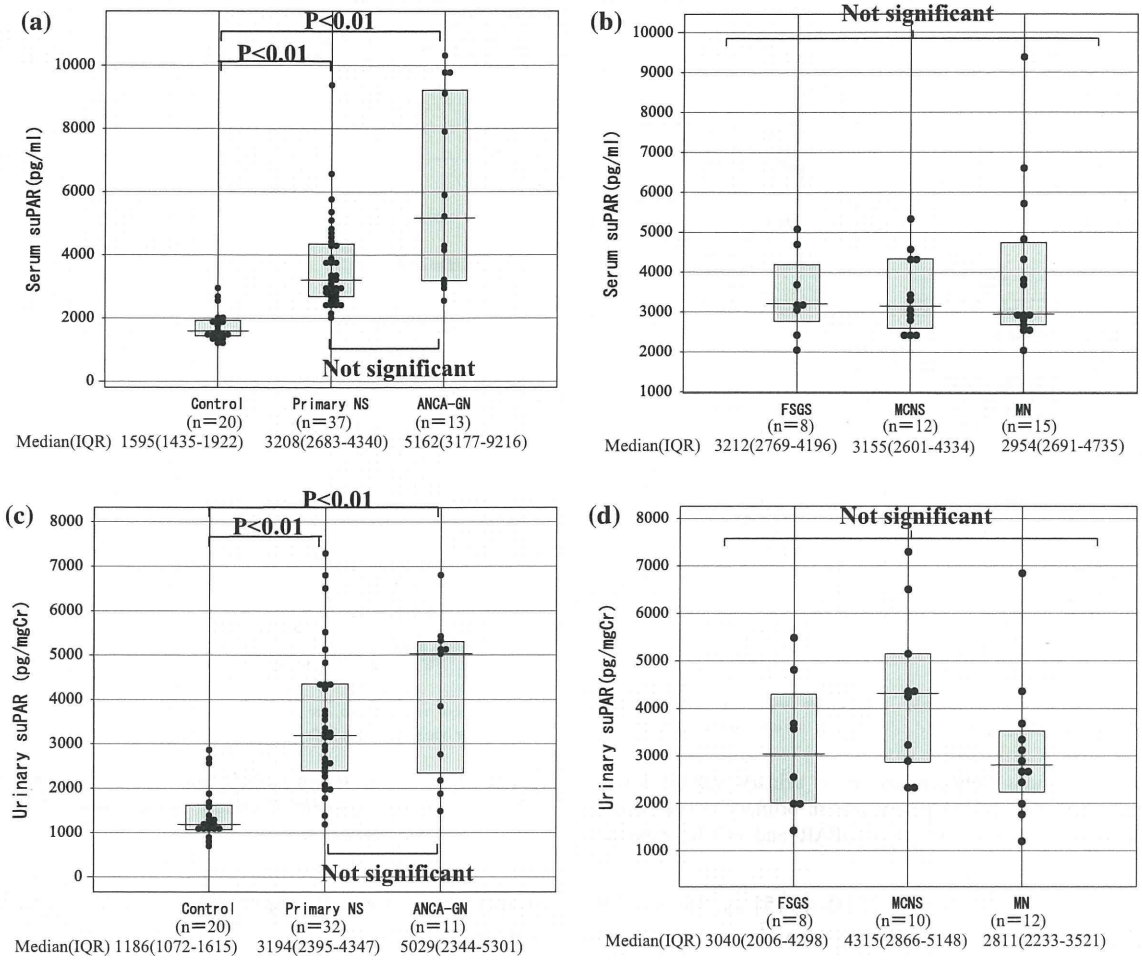

Fig. 1 Comparison of serum and urinary suPAR levels before therapy **a** Serum suPAR levels before therapy were significantly higher in patients with primary NS or ANCA-GN than in normal controls ($p < 0.01$). On the other hand, no significant differences were noted in pretreatment serum suPAR levels between primary NS and ANCA-GN. **b** There were no significantly differences among patients with FSGS, MCNS, MN in serum suPAR levels before therapy. **c** Urinary suPAR levels before therapy were significantly higher in patients with Primary NS or ANCA-GN than in normal controls ($p < 0.01$). On the other hand, no significant differences were noted in pretreatment urinary suPAR levels between primary NS and ANCA-GN. **d** There were no significant differences among patients with FSGS, MCNS, MN in urinary suPAR levels before therapy

supplement figure 1a) between s- and u-suPAR, but not in the ANCA-GN group ($n = 11$, $\rho = 0.245$, $p = 0.467$, supplement figure 1b).

Regarding the relationships between pretreatment u-suPAR and clinical parameters (primary NS, Fig. 2; ANCA-GN, Fig. 3), a significant positive correlation with CRP was noted in the ANCA-GN group ($n = 11$, $\rho = 0.656$, $p = 0.028$, Fig. 3f). U-suPAR positively correlated with UP in the primary NS group ($n = 32$, $\rho = 0.501$, $p = 0.003$, Fig. 2e), but inversely correlated with UP in the ANCA-GN group ($n = 11$, $\rho = -0.864$, $p = 0.001$, Fig. 3d).

Alterations in UP, serum suPAR, and urinary suPAR after immunosuppressive therapy

S-suPAR was measured for 2 months after the initiation of immunosuppressive therapy in 7 FSGS, 9 MCNS, 9 MN, and 2 MPGN patients. The relationship between changes in s-suPAR over 2 months of immunosuppressive therapy (Δ2M s-suPAR) and the treatment response of NS was investigated in these patients. Four MN, 1 FSGS (NOS), and 1 MPGN patients were intractable for therapy. S-suPAR during the 2-month period significantly decreased in MCNS ($n = 9$, 2996 [IQR 2677–4315] pg/mL before treatment vs. 2601 [IQR 2392–2882] pg/mL at 2 months of treatment, $p = 0.011$, Fig. 4a), and in non-intractable NS ($n = 21$, 2996 [IQR 2664–3739] vs. 2638 [IQR 2453–3242] pg/mL, $p = 0.023$). In contrast, s-suPAR during the 2-month period significantly increased in intractable NS ($n = 6$, 4249 [IQR 2939–6580] vs. 5267 [IQR 3474–8243] pg/mL, $p = 0.046$). No significant changes were noted in s-suPAR throughout the 2-month period in FSGS or MN (FSGS, $n = 7$, 3208 [IQR 2610–3605] vs. 2840 [IQR 2549–4668] pg/mL, $p = 0.499$,

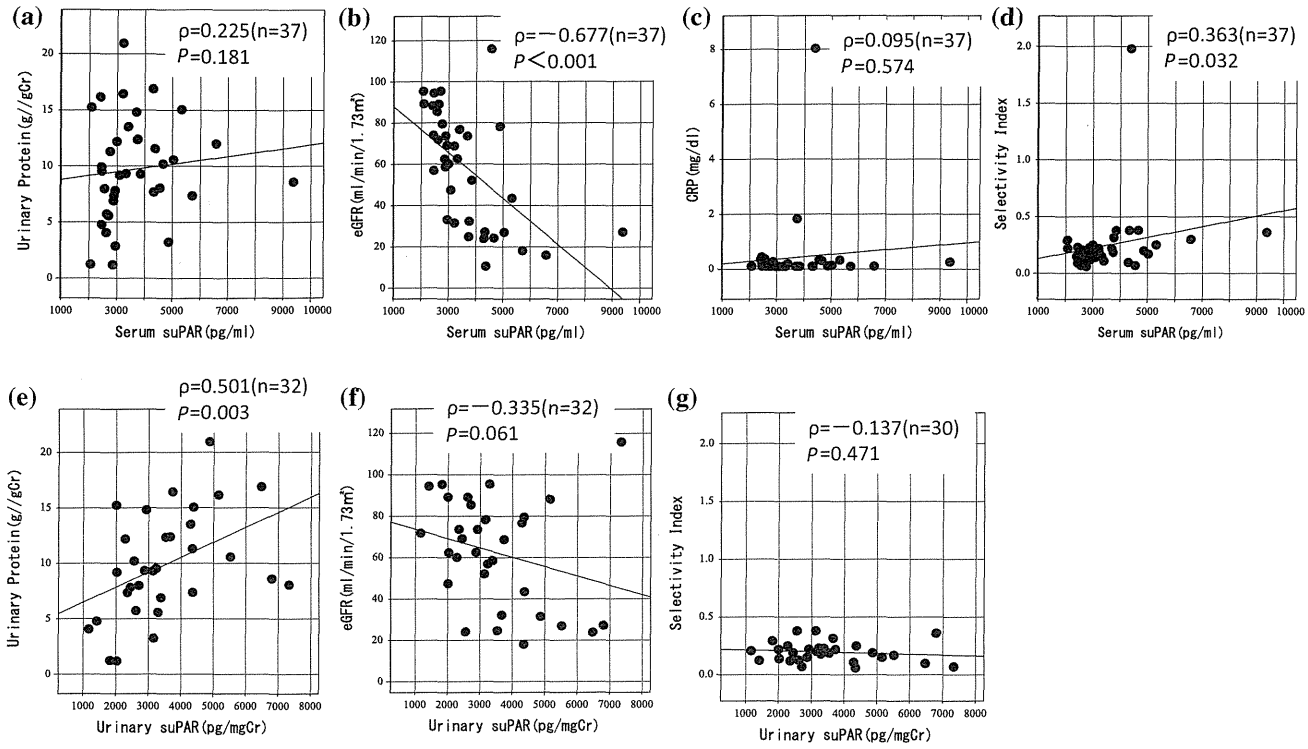

Fig. 2 The correlations between serum or urinary suPAR levels before therapy and the clinical parameters in primary NS. **a** Serum suPAR and urinary protein, **b** serum suPAR and eGFR, **c** serum suPAR and CRP, **d** serum suPAR and selectivity index, **e** urinary suPAR and urinary protein, **f** urinary suPAR and eGFR, **g** urinary suPAR and selectivity index

Fig. 4b; MN, $n = 9$, 2954 [IQR 2810–4545] vs. 3898 [IQR 2779–5227] pg/mL, $p = 0.110$, Fig. 4c). However, there were no significant changes in eGFR and CRP in these cases (data not shown). On the other hand, UP was significantly decreased in FSGS, MCNS, MN (supplement figure 2), non-intractable NS ($n = 21$, 11.30 [IQR 7.24–15.10] vs. 0.04 [IQR 0.01–0.14] g/gCr, $p < 0.001$), and intractable NS ($n = 6$, 8.95 [7.82–10.20] vs. 2.61 [1.91–3.94] g/gCr, $p = 0.028$) after therapy.

In addition, u-suPAR was measured for 2 months after the initiation of immunosuppressive therapy in 7 FSGS, 7 MCNS, 6 MN, and 2 MPGN patients. The relationship between changes in u-suPAR over 2 months of immunosuppressive therapy (Δ2M u-suPAR) and the treatment response of NS was investigated in these patients. Three MN, 1 FSGS (NOS), and 1 MPGN patient were intractable for therapy. U-suPAR significantly decreased during the 2-month period in MCNS ($n = 7$, 4341 [IQR 2478–4953] vs. 2148 [IQR 1568–2882] pg/mgCr, $p = 0.018$, Fig. 4d) or in non-intractable NS ($n = 17$, 3379 [IQR 2331–4348] vs. 2552 [IQR 1834–3148] pg/mgCr, $p = 0.039$). However, no significant changes were noted in u-suPAR throughout the 2-month period in FSGS or MN (FSGS, $n = 7$, 2550 [IQR 2001–3682] vs. 2925 [IQR 1969–4318] pg/mgCr, $p = 0.735$, Fig. 4e; MN, $n = 6$, 3041 [IQR 2441–3664] vs. 3023 [IQR 2552–3774] pg/mgCr, $p = 0.345$, Fig. 4f).

Significant factors affecting Δ2M s-suPAR and 2M suPAR

When we examined the factors affecting Δ2M s-suPAR using multiple regression analysis, the therapeutic response was identified as a significant factor, whereas CRP and eGFR were not (data shown in supplement Table 1). Moreover, a significant positive correlation was observed between s-suPAR at 2 months of treatment (2M s-suPAR) and UP at 2 months of treatment ($n = 27$, $\rho = 0.518$, $p = 0.006$). The multiple regression analysis suggested that the factors affecting 2M s-suPAR were the therapeutic response and eGFR, with the therapeutic response being the most influential factor (data shown in supplement Table 2).

What is the useful parameter for defining the new standard of therapeutic response at the early phase after therapy in Japanese patients with NS?

ROC analyses were performed to define the new standard of therapeutic response of NS at the early phase after therapy which was consistent with the conventional Japanese definition of therapeutic response at 6 months after therapy. The patients with primary NS could be divided into intractable NS group and non-intractable NS group

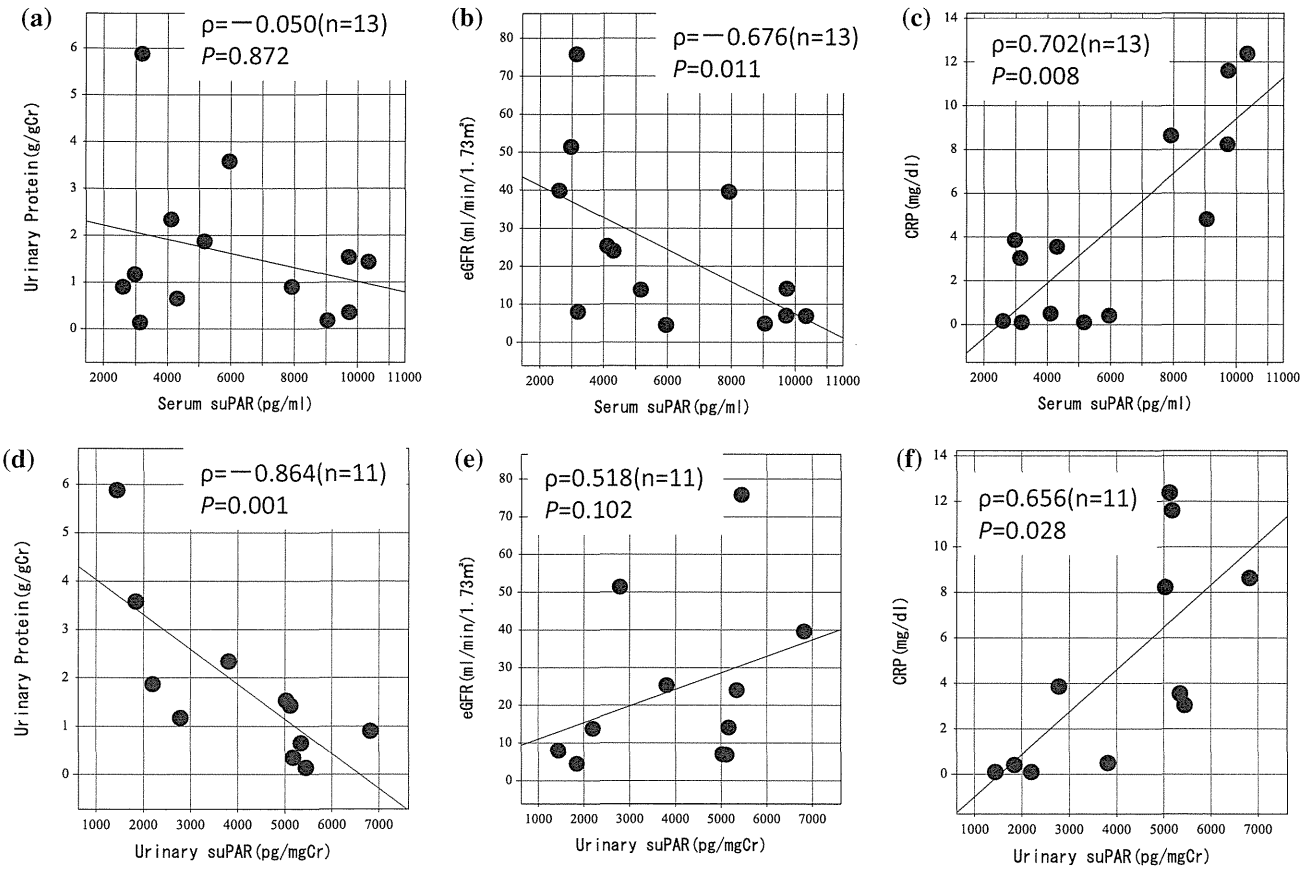

Fig. 3 The correlations between serum or urinary suPAR levels before therapy and the clinical parameters in ANCA-GN. **a** Serum suPAR and urinary protein, **b** serum suPAR and eGFR, **c** serum suPAR and CRP, **d** urinary suPAR and urinary protein, **e** urinary suPAR and eGFR, **f** urinary suPAR and CRP

within 2 months after therapy by using UP at 2 months after therapy (2M UP), s-suPAR at 2 months after therapy (2Ms-suPAR) or changes in u-suPAR during 2 months after therapy (Δ2M u-suPAR) (Table 2).

The most useful parameter for differentiating FSGS from MCNS

Comparisons of the areas under the curves of receiver operating characteristic analyses (AUC-ROCs) of clinical parameters in differentiating FSGS from MCNS are shown in Table 2. To differentiate FSGS from MCNS, Δ2M s-suPAR was found to be the most useful predictor, whereas UP was not (The cut-off values shown in Table 2, and ROC curves of suPAR and UP in supplement figures 3, 4.)

Clinical severity of and crescentic formation in ANCA-GN were associated with s-suPAR

In the ANCA-GN group, a significant positive correlation was noted between s-suPAR before immunosuppressive therapy and clinical severity ($n = 13$, $\rho = 0.651$, $p = 0.016$) or the percentage of crescentic formation ($n = 10$, $\rho = 0.770$, $p = 0.009$), whereas no correlation was noted between pretreatment u-suPAR and clinical severity ($n = 12$, $\rho = 0.300$, $p = 0.344$) or the percent of crescentic formation ($n = 9$, $\rho = 0.150$, $p = 0.700$).

Discussion

This single-center retrospective cohort study clarified the clinical significance of s- and u-suPAR in Japanese untreated primary NS and ANCA-GN patients. This study assessed u-suPAR in Japanese adult patients with NS for the first time. Pretreatment s-suPAR was significantly higher in the primary NS and ANCA-GN groups, whereas no significant differences were noted among the disease types of primary NS, suggesting that FSGS cannot be differentiated from other disease types of primary NS based on pretreatment s-suPAR. S-suPAR was previously shown to be significantly higher in primary FSGS only [1, 4], whereas, similar to our results, no significant differences were noted between FSGS and other types of

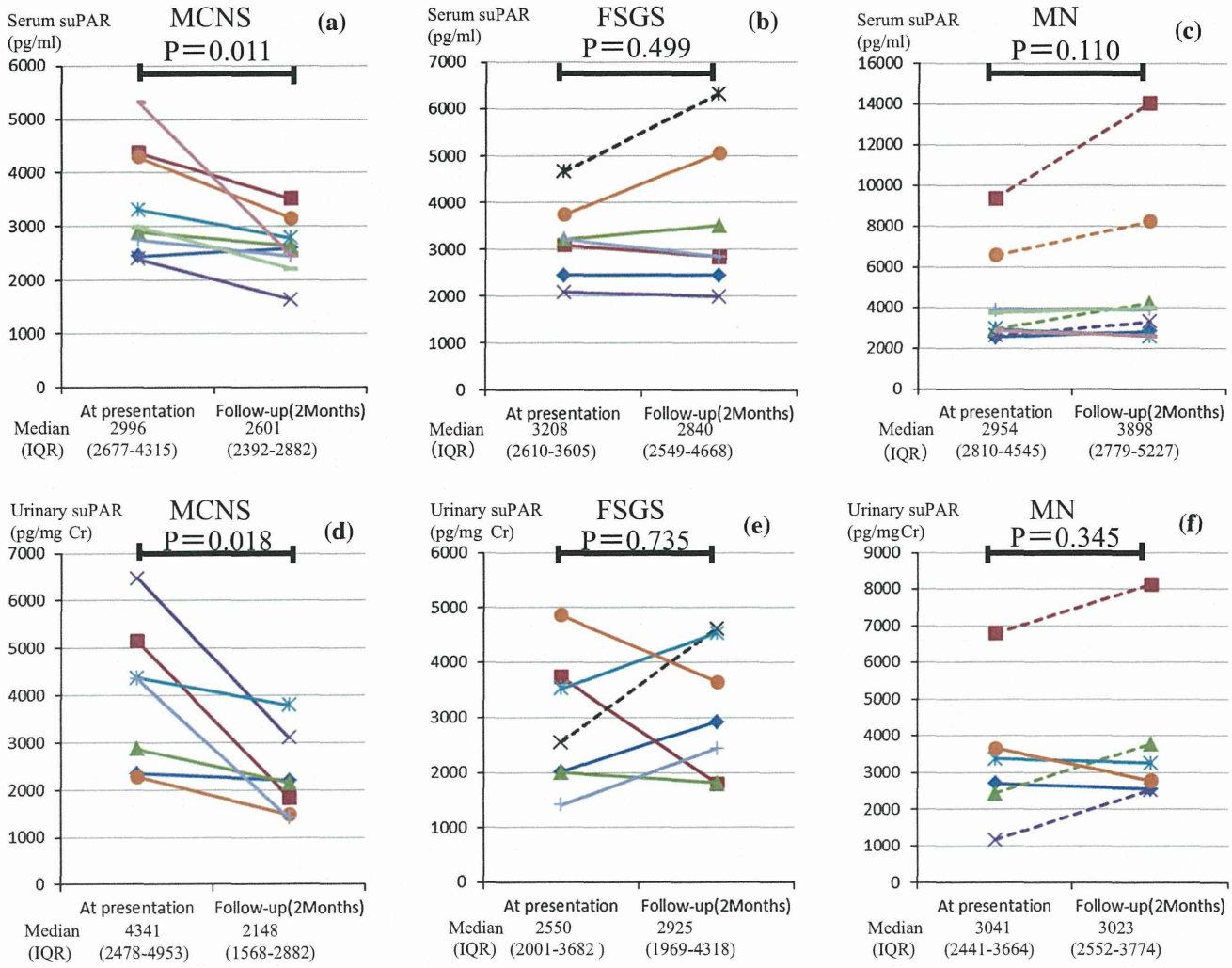

Fig. 4 The changes of serum and urinary suPAR levels after therapy among the disease types of primary NS *Solid lines* are non-refractory cases. *Dotted lines* are the refractory cases. In patients with MCNS (**a**) (**d**), serum and urinary suPAR levels significantly decreased. In patients with FSGS (**b**) (**e**) and MN (**c**) (**f**), serum and urinary suPAR levels did not change

primary NS by other studies [6–8, 12, 13]. The influence of immunosuppressive therapy on s-suPAR was considered to be the cause of this inconsistency [3, 4, 14, 15]. However, as shown in our results, comparisons of pretreatment s-suPAR, which may be specific to each disease, revealed no significant differences among the diseases.

Since the molecular weight of the main fragment of suPAR is 22 kDa and it passes through the glomerular filtration barrier, s-suPAR may be influenced by GFR or increase due to nonspecific inflammation [16–18]. No significant differences were noted in eGFR or CRP before therapy among the disease types of primary NS, suggesting that these did not influence the comparison of pretreatment s-suPAR in this study. On the other hand, pretreatment eGFR was lower and pretreatment CRP was higher in the ANCA-GN group than in the primary NS group, suggesting that renal dysfunction and inflammation led to s-suPAR being higher in the ANCA-GN group than in the primary NS group. We then investigated the relationships between pretreatment s-suPAR and clinical parameters. A strong inverse correlation was noted between s-suPAR and eGFR in FSGS, as previously reported [3, 4]. Furthermore, an inverse correlation with eGFR was also found in all primary NS and MN, which was consistent with recent findings [6–8]. In contrast, no correlation with any of the clinical parameters was noted in MCNS. These findings may suggest that s-suPAR is associated with renal dysfunction in primary NS, particularly in FSGS and MN patients with a poor renal prognosis.

On the other hand, s-suPAR was positively correlated with CRP and inversely correlated with eGFR in the ANCA-GN group. CRP and eGFR were identified as

Table 2 Receiver operating characteristic (ROC) analysis of clinical parameters as predictors of intractable NS or FSGS

Clinical parameters	AUC-ROC	Cut-off value	Sensitivity (=specificity)	*p*	*n*
The diagnostic performance of differentiating intractable NS from non-intractable NS					
2M s-suPAR	0.913	3,373 (pg/mL)	0.762	0.002	27
Δ2M s-suPAR	0.881	−189 (pg/mL)	0.833	0.005	27
2M u-suPAR	0.776			0.066	22
Δ2M u-suPAR	0.906	−127 (pg/mgCr)	0.800	0.007	22
2M UP	0.968	0.97 (g/gCr)	0.952	0.001	27
Δ2M UP	0.833	−6.97 (g/gCr)	0.762	0.014	27
2M eGFR	0.730			0.091	27
Δ2M eGFR	0.579			0.560	27
The diagnostic performance of differentiating FSGS from MCNS					
2Ms-suPAR	0.698			0.186	16
Δ2M s-suPAR	0.905	−268 (pg/mL)	0.857	0.007	16
2M u-suPAR	0.694			0.225	14
Δ2M u-suPAR	0.816	−718 (pg/mgCr)	0.714	0.048	14
2M UP	0.675			0.244	16
Δ2M UP	0.508			0.958	16
2M eGFR	0.587			0.560	16
Δ2M eGFR	0.651			0.315	16

The cut-off value is at the point where the specificity equal to the sensitivity

NS nephrotic syndrome, *FSGS* focal segmental glomerulosclerosis, *MCNS* minimal change nephrotic syndrome, *s-* serum, *u-* urinary; *2M* at 2 months after therapy, *Δ2M* change during 2 months after therapy, *UP* urinary protein, *AUC-ROC* the area under the curve of receiver operating characteristic analysis

factors that influence vital and renal prognoses in a study on Japanese ANCA-GN, and these were found to be important evaluation items to judge the clinical severity of ANCA-GN. Based on these findings, we assumed that s-suPAR was associated with the clinical severity of ANCA-GN, suggesting that s-suPAR is a useful clinical severity marker of ANCA-GN. In addition, a positive correlation was noted between pretreatment s-suPAR and crescent formation in this study. Baraldi et al. [19] detected the strong expression of β3 integrin on crescent cells, podocytes, and Bowman's capsular epithelial cells in RPGN patients, and suggested the involvement of β3 integrin in the mechanism underlying crescent formation. Thus, it was also assumed that s-suPAR activated β3 integrin on podocytes and Bowman's capsular epithelial cells, and this process was part of the mechanism underlying crescent formation in ANCA-GN as well as crescentic FSGS [4].

Regarding s-suPAR as a clinical marker, we also investigated the time-course of s-suPAR over 2 months of immunosuppressive therapy because s-suPAR over 3,000 pg/mL was previously shown to activate podocyte β3 integrin [1]. Although intractable and non-intractable cases could not be differentiated based on s-suPAR over 3,000 pg/mL before immunosuppressive therapy, which is consistent with previous findings [7], 3,373 pg/mL or higher at 2 months may be used for a marker to judge responses to treatments. These changes in s-suPAR after therapy were not significantly influenced by changes in eGFR or CRP in this study. Moreover, 2M s-suPAR was positively correlated with 2M UP and a cut-off value of 2M s-suPAR to differentiate intractable cases was close to the s-suPAR level (3,000 pg/mL) in the first study [1]. Reiser et al. may have observed this phenomenon in primary FSGS cases treated with immunosuppressive drugs.

In our study, differentiating between FSGS and MCNS was possible based on changes in s-suPAR over 2 months of immunosuppressive therapy. Huang et al. [4] compared s-suPAR before immunosuppressive therapy with that after 78 weeks of therapy on average in primary FSGS patients, and observed a significant decrease in complete remission cases, no change in incomplete remission cases, and a significant elevation in ineffective cases. These findings revealed changes in s-suPAR that corresponded to the responses to treatments. Similar to our results, they could not predict therapeutic responses in FSGS patients based on s-suPAR before immunosuppressive therapy [4]. On the other hand, no significant change was noted in s-suPAR over the 2-month period in this study. Considering that the follow-up period was markedly longer (78 weeks on average) in their study than in ours (8 weeks), the 2-month period may have been too short to observe a reduction in