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NPHS2 mutation analysis shows genetic heterogeneity of steroid-resistant nephrotic syndrome and low post-transplant recurrence

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***NPHS2* mutation analysis shows genetic heterogeneity of steroid-resistant nephrotic syndrome and low post-transplant recurrence.**

Background. Mutations of *NPHS2* are causative in familial autosomal-recessive (AR) and sporadic steroid-resistant nephrotic syndrome (SRNS). This study aimed to determine the spectrum of *NPHS2* mutations and to establish genotype-phenotype correlations.

Methods. NPHS2 mutation analysis was performed in 338 patients from 272 families with SRNS: 81 families with AR SRNS, 172 patients with sporadic SRNS, and 19 patients with diffuse mesangial sclerosis (DMS).

Results. Twenty-six different pathogenic *NPHS2* mutations were detected, including 13 novel mutations. The mutation detection rate was 43% for familial AR and 10.5% for sporadic SRNS, confirming genetic heterogeneity. No pathogenic *NPHS2* mutations were found in DMS patients. Age at onset in patients with two pathogenic mutations was earlier, especially in cases with frameshift, truncating, and the R138Q missense mutations. Patients with only one *NPHS2* mutation or variant had late-onset NS. Triallelic inheritance was observed in one patient with a homozygous R138Q mutation and a *de novo NPHS1* mutation. Among 32 patients with two *NPHS2* mutations who underwent kidney transplantation, only one developed late recurrence of focal segmental glomerulosclerosis (FSGS). Among 25 patients with sporadic SRNS and post-transplantation recurrence, we detected a heterozygous *NPHS2* mutation in one case, and heterozygous variants/polymorphisms in 3 cases.

Conclusion. Patients with two pathogenic *NPHS2* mutations present with early-onset SRNS and very low incidence of post-transplantation recurrence. Heterozygous *NPHS2* variants may play a role in atypical cases with mild, late-onset course, and recurrence after transplantation.

Key words: *NPHS2* gene, podocin, steroid-resistant nephrotic syndrome, diffuse mesangial sclerosis.

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Nephrotic syndrome (NS) is frequently seen as an idiopathic primary disease that represents a heterogeneous group of glomerular disorders occurring mainly in children. It is generally divided into steroid-sensitive and steroid-resistant forms, depending upon the patient's response to steroid therapy. While most patients show a favorable outcome after steroid treatment, 10% of children with idiopathic NS do not respond to steroids or to any other immunosuppressive therapy, and progress to end-stage renal disease (ESRD). Renal histology typically shows focal segmental glomerulosclerosis (FSGS). Furthermore, in approximately one third of transplanted patients, the clinical course is complicated by a recurrence of proteinuria, histologically characterized by reappearance of FSGS in the transplanted kidney, which may ultimately compromise allograft function [1]. Several pathogenic factors have been postulated in these cases of recurrent disease after transplantation, among these a proteinuric circulating permeability factor, so far unidentified, produced in the setting of T-cell dysfunction [2, 3].

Diffuse mesangial sclerosis (DMS) is a separate histologic entity, clinically presenting with SRNS in early childhood and rapidly leading to ESRD in the first years of life. Renal histology shows enhanced production of mesangial matrix, thickening of the glomerular basement membrane (GBM), and podocyte hypertrophy.

Over the last decade, mutations in genes encoding podocyte proteins have been identified in several forms of hereditary NS. Mutations in the Wilm's tumor suppressor gene *WT1* were discovered in patients with Denys-Drash, Frasier syndrome, and DMS [4–7]. *NPHS1,* which encodes nephrin, has been identified as the causative gene in congenital NS of the Finnish type (CNF) [8]. Mutations in *ACTN4* encoding α-actinin-4 were discovered in several families with autosomal-dominant FSGS, a form of hereditary NS with late onset and progression to ESRD [9]. Very recently, a heterozygous splice-site mutation

in *CD2AP*, encoding CD2-associated protein, a component of the glomerular filtration complex, was described in two unrelated patients with primary FSGS, pointing to additional genes which might be involved in human susceptibility to glomerular disease [10]. By positional cloning, a novel podocyte-specific protein, podocin, encoded by *NPHS2* on chromosome 1q25-31 and associated with autosomal-recessive forms of SRNS, was described by Boute et al [11]. Podocin is a 383-amino acid membrane protein that shares sequence homology with proteins of the band-7 stomatin family. It has a predicted hairpin-like structure and is expressed exclusively at the slit diaphragm, with both protein termini on the cytosolic side [12]. Podocin has been shown to accumulate in an oligomeric form in the slit diaphragm, where it interacts with CD2AP and nephrin [13]. It has been postulated that podocin may serve a scaffolding function in lipid rafts, and it has also been shown to direct the targeting of nephrin to lipid rafts, where it augments nephrin signaling [14–16].

NPHS2 mutations were first detected in familial autosomal-recessive SRNS, which manifests early in childhood and presents histologically with FSGS [11]. Thereafter, *NPHS2* mutations were also identified in sporadic cases of SRNS [17–19], late onset FSGS [20], and, surprisingly, in a few patients with recurrence of proteinuria after renal transplantation [21]. Since the discovery of podocin, we have performed mutational analysis and clinical follow-up of a large cohort of patients with either familial or sporadic SRNS originating mainly from France and North African countries. We present our results, correlating the age of onset and likelihood of post-transplantation recurrence in these patients with the nature of the underlying *NPHS2* mutation/variant, and compare our results with other published series [17–24].

METHODS

Patients

A total of 338 patients belonging to 272 families were included in the present study. They originated from a large number of countries worldwide, most of them, however, from Europe (mainly France) and from North Africa. Of these, 319 presented with SRNS, defined as lack of response to four weeks of treatment with prednisone followed by three bolus injections of methylprednisolone. Among them, 147 patients belonging to 81 families (44 being consanguineous) were classified as autosomal-recessive (AR) SRNS, defined as SRNS in families with either two or more affected children, or one (or more) affected individual in consanguineous families. This group included 16 families that were previously described in our initial study [11]. One hundred seventy-two patients presented with sporadic SRNS. Other distant family members were affected with proteinuria or NS in 7 cases. As the patterns of inheritance in these families were not suggestive of autosomal-recessive disease, the index cases were included in the group of sporadic SRNS. In addition, 19 patients (of 19 families) with DMS were also included in the present study.

Mean age at manifestation of NS was recorded in 286 patients and was 57.6 $\pm$ 6.8 months in AR SRNS (N = 107), whereas it was higher in sporadic cases (102.9 $\pm$ 8.9 months; $N = 169$), especially in the cases with recurrence after transplantation (134.3 $\pm$ 27.3 months; N = 24). Manifestation was early in the patients affected by DMS (22.1 $\pm$ 5.8 months; $N = 10$). In total, as most of the patients were recruited through pediatric nephrology departments, only 37 developed NS after 15 years (4 patients with AR SRNS and 33 patients with sporadic SRNS, 7 of them with post-transplantation recurrence).

For 261 patients, the results of at least one kidney biopsy were provided to us (106 patients with AR SRNS and 155 sporadic cases). These evaluations showed that 65 patients had predominantly minimal changes (23 patients with AR SRNS, 42 sporadic cases) and 172 patients had FSGS (68 patients with AR SRNS, 104 sporadic cases). In a few cases, mesangial proliferation was observed (15 patients with AR SRNS, 9 sporadic cases) without glomerulosclerosis.

Information on renal transplantation was available for 115 patients with AR SRNS and 170 patients with sporadic SRNS. Transplantation was performed in 44 of the 115 patients with AR SRNS, and no early recurrence was observed in these cases. However, one patient developed proteinuria and FSGS 2 years after receiving an allograft. Among the group of sporadic SRNS, 74 underwent kidney transplantation and 25 of them had a recurrence of proteinuria. In 16 of these, early recurrence was diagnosed immediately after transplantation. Six patients showed a recurrence 14 days to 18 months after transplantation. No post-transplant data were available for 3 patients.

Genotyping and haplotype analysis

Peripheral blood samples were obtained after informed consent of the patients or their parents. Genomic DNA was extracted according to standard laboratory protocols. Five polymorphic microsatellite markers (D1S3758, D1S3760, D1S215, D1S3759, D1S2883) spanning 1.1 cm and flanking the *NPHS2* locus were tested. Genotyping was performed after PCR amplification and electrophoresis as previously described in Fuchshuber et al [25]. Cyrillic 2.1 (Cherwell Scientific, Oxford, UK) was used to construct pedigrees and to perform haplotype analysis.

Mutation screening

Mutation analysis for *NPHS2* was carried out by single-strand conformation polymorphism (SSCP) analysis for

exons 2–7, as described previously in Boute et al [11], and by direct sequence analysis for exons 1 and 8. Exons of the *NPHS1* gene were amplified by PCR using flanking intronic primers [26] and subsequently sequenced. Direct sequencing was carried out using the Big Dye-terminator method (Applied Biosystems, Foster City, CA, USA). Sequences were evaluated with the Sequencher software (Gene Codes, Ann Arbor, MI, USA). Segregation analysis of mutations in families was performed by direct sequencing, SSCP analysis, or specific restriction enzyme digestion.

Genotyping by single-base extension (SBE) and electrophoresis

In order to discriminate mutations from polymorphisms, all detected missense mutations were screened in 320 control chromosomes by single-base extension using SNaPshot Multiplex kit (Applied Biosystems). We expanded the control cohort to 75 individuals (150 chromosomes) of African descent for the screening of the A242V variant. Exons were amplified using flanking intronic primers. The PCR products from 4 to 6 exons were then mixed with 1.5 μL of ExoSAP-IT (Amersham Bioscience) and incubated for 1 hour at 37°C to remove primers and dNTP, followed by 15 minutes at 80°C for enzyme inactivation. Primer extension reactions [27] were performed according to manufacturer's protocol. To clean up the single-base extension reaction, one unit of shrimp alkaline phosphatase (Amersham Bioscience) was added to the reaction and then incubated for 1 hour at 37°C, followed by 15 minutes at 80°C for enzyme inactivation. The extension products were denatured for 5 minutes at 95°C, and electrophoresis was performed using ABI Prism 373 A DNA Sequencer (Applied Biosystems). The results were analyzed using Genescan Analysis and Genotyper (Applied Biosystems).

Statistical analyses

Values reported are the mean ± standard error of the mean (SEM). Statistical comparisons were performed using one-way analysis of variance (ANOVA). P values of < 0.05 were considered significant.

RESULTS

Linkage analysis

In 62 families suggestive of AR SRNS, linkage analyses were performed with markers flanking the *NPHS2* gene locus on chromosome 1q25-31. Results were compatible with linkage to this locus in 36 families, and pathogenic *NPHS2* mutations were identified in 25 of them. In the remaining 11 families (all consanguineous, 10 with only one affected child), linkage was compatible as evidenced by homozygosity for the polymorphic makers flanking *NPHS*2, but no pathogenic *NPHS2* mutations were identified. Linkage to the *NPHS2* locus was excluded in 26 families, and we performed *NPHS2* mutation screening in 17 of these families in parallel. No *NPHS2* mutations were detected, as was expected, and further mutation analysis in the 9 additional families was not performed.

Homozygous, compound heterozygous, and single heterozygous pathogenic *NPHS2* mutations

NPHS2 mutation screening was thus performed in 322 patients from 263 families. Pathogenic mutations were defined as variants that (*1*) were not found in 320 healthy control chromosomes, (*2*) segregated with the disease in families, and (*3*) were predicted to severely alter protein sequence and/or expression levels. Homozygous or compound heterozygous mutations were identified in 73 patients (42 families), of whom 62 patients presented clinically with AR SRNS (31 families), and 11 with sporadic SRNS. We did not identify pathogenic *NPHS2* mutations in any patient with isolated DMS (Table 1).

In addition, only one pathogenic *NPHS2* mutation was identified in 2 patients from one family with AR SRNS and in 6 patients with sporadic SRNS. The entire coding region and all exon-intron boundaries of *NPHS2* were sequenced in order to identify the second mutation, but yielded no additional mutation. Although some mutations may have been missed, such as heterozygous deletions, intronic mutations, or mutations in regulatory regions, it is unlikely that they could account for all the unidentified mutations. In addition to these 8 patients, 7 patients from 4 families (3 AR SRNS and 1 sporadic case) presented with compound heterozygosity of one pathogenic *NPHS2* mutation and the R229Q polymorphism on the other allele.

The detection rate of *NPHS2* mutations in the homozygous or compound heterozygous states for AR SRNS was 38% (31 out of 81 families studied by mutation screening and/or linkage analysis). On the contrary, mutation detection rate for sporadic SRNS was five times lower at 6.4% (11 affected patients of 172 patients studied). If patients with heterozygous pathogenic *NPHS2* mutation with or without R229Q in compound heterozygosity were also included, the mutation detection rates rose to 43% for AR SRNS and to 10.5% for sporadic cases, respectively.

In total, 26 different *NPHS2* mutations were detected in the present study; among these, 13 were novel mutations (Table 2). These newly identified mutations include missense, nonsense, frameshift and splice-site mutations, and one in-frame deletion of 9 nucleotides, but no large or complete gene deletions. Some mutations were found in several unrelated families. In particular, the R138Q mutation (in exon 3) was observed in 32% of all affected alleles. A large majority of mutations were located in exons 4, 5, and 7, encoding the C-terminal part of podocin. *NPHS2* missense mutations cluster in the early

Table 1. Overview of genotypic and phenotypic data

	AR SRNS[a]	Sporadic SRNS[a]	DMS[a]
No. of patients (families)	147 (81)	172 (172)	19 (19)
Two *NPHS2* pathogenic mutations	62 (31)	11 (11)	0 (0)
One *NPHS2* pathogenic mutation	2 (1)	6 (6)	0 (0)
One *NPHS2* mutation + R229Q	6 (3)	1 (1)	0 (0)
Mutation detection rate (in total)	43%	10.5%	0%
Homozygous R229Q polymorphism	5 (3)	2 (2)	0 (0)
Heterozygous R229Q polymorphism	0 (0)	11 (11)	2 (2)
Variants in heterozygous state	3 (2)	4 (4)	1 (1)
A242V polymorphism	2 (2)	4 (4)	1 (1)
Age at onset (all)	57.6 ± 6.8 months $N = 107$	102.9 ± 8.9 months $N = 169$	22.1 ± 5.8 months $N = 10$
Age at onset (with two pathogenic *NPHS2* mutations)	41.2 ± 5.9 months $N = 63$		
Age at onset (without two pathogenic *NPHS2* mutations)	69.9 ± 11.5 months $N = 55$; $P = 0.019$[b]	108.3 ± 9.4 months $N = 158$; $P < 0.001$[b]	

[a]Numbers in parentheses refer to affected families.
[b]*P* value: age at onset is compared with patients bearing two pathogenic mutations.

Table 2. Summary of mutations, variants, and polymorphisms

Exon	Nucleotide change	Effect on protein	Status (number of unrelated patients)	Reference(s)
Pathogenic mutations				
1	85 G>A	A29T	het (1)	Present study
1	104_105insG	G35fsX69	c het (1)	[11]
1	274 G>T	G92C	c het (1)	[11]
2	275-2 A>C	Splice	c het (1)	Present study
2	353 C>T	P118L	hom (1), c het (1)	Present study
3	412 C>T	R138X	hom (1)	[11, 18, 23]
3	413 G>A	R138Q	hom (12), c het (7), het (1)	[11, 17, 18, 20, 22]
3	419delG	G140fsX180	hom (1), c het (1)	[11, 17, 18]
4	467delT	L156fsX180	hom (1)	Present study
4	467_468insT	L156fsX166	hom (1), c het (1)	[17, 18, 22]
4	479 A>G	D160G	hom (1)	[11]
4	502 C>A	R168S	c het (1)	Present study
4	502 C>T	R168C	het (1)	Present study
4	503 G>A	R168H	hom (1), c het (1), het (1)	Present study
5	538 G>A	V180M	hom (3), c het (2)	[11, 17, 18]
5	555delT	F185fsX186	c het (1)	[19]
5	622 G>A	A208T	het (2)	Present study
5	705_713del9	L236_R238del	hom (1)	Present study
5	714 G>T	R238S	hom (1), c het (1)	Present study
6	779 T>A	V260E	hom (4)	Present study
7	851 C>T	A284V	hom (1), het[a] (2)	[17, 20]
7	855_856delAA	Q285fsX302	hom (1), c het (4)	[11, 17, 20]
7	862 G>A	A288T	het[a] (1)	[20]
7	871 C>T	R291W	het[a] (1)	[11, 17, 20]
8	964 C>T	R322X	c het (1)	Present study
8	976_977insA	T326fsX345	het (1)	Present study
Variants of unknown significance				
1	59 C>T	P20L	hom[b] (1), het (1)	[11, 19]
1	182 C>T	A61V	het[c] (2)	Present study
4	514 C>G	L172V	het (1)	Present study
5	709 G>C	E237Q	het (2)	Present study
Nonsilent polymorphisms in the coding sequence				
5	686 G>A	R229Q	hom (5), c het (4), het (13)	[17, 20]
5	725 C>T	A242V	het (7)	Present study

Abbreviations are: hom, homozygous mutation; het, single heterozygous mutation; c het, compound heterozygous mutation.
[a]Heterozygous mutation with the R229Q polymorphism on the second allele; [b]also with the R168H mutation on the same allele; [c]one patient with SMD, the other also bearing the A242V polymorphism.

C-terminal cytoplasmic domain of the podocin protein, the region conserved among members of the stomatin protein family (see Fig. 1). Only one mutation (P118L) was detected in the membrane domain, and 4 (A29T, G35fsX59; G92C; 275–2 A>C) in the N-terminal part of podocin. No missense mutations occurred in predicted N- or O-glycosylation, phosphorylation, or myristolation sites.

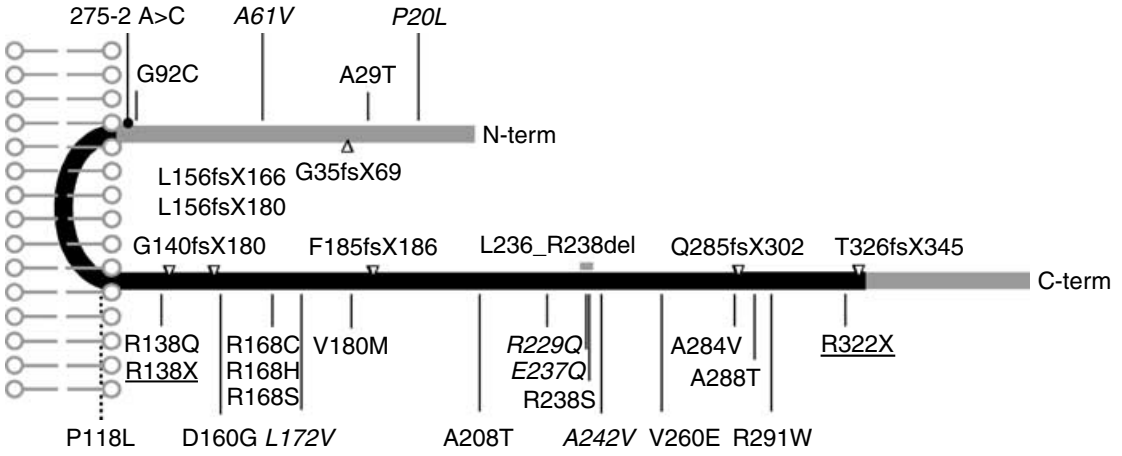

Fig. 1. Podocin structure and distribution of mutations. Predicted two-dimensional protein structure of podocin with both termini on the cytosolic side. The region of homology with stomatin is depicted in black. Variants of unknown significance and polymorphisms are shown in italics, nonsense mutations are underlined, and small insertions/deletions are depicted by arrowheads. One splice-site mutation (275–2 A>C) was identified affecting the acceptor splice-site of intron 1.

Sequence variations with unknown phenotypic effects and polymorphisms in *NPHS2*

Several amino acid substitutions (P20L, A61V, L172V, E237Q, and A242V) were identified that either do not markedly alter the predicted protein structure (i.e., conservative amino acid change), and/or affect residues that are not highly conserved among members of the stomatin family. These amino acid substitutions were detected in the heterozygous state in AR and sporadic cases of SRNS, as well as in two DMS patients. Subsequently, the frequencies of these sequence changes were screened by SNaPshot analysis in a control cohort of 320 chromosomes. Screening results revealed that none of the healthy controls showed a P20L, A61V, or L172V sequence variant, indicating that these substitutions are probably not polymorphisms. However, in support of a minor role of these variants, a heterozygous L172V variant was identified in an AR SRNS family without linkage to the *NPHS2* gene locus. A homozygous P20L variant was sequenced along with a homozygous pathogenic R168H mutation in a consanguineous family with two affected children. Finally, the A61V variant was observed in a case of sporadic SRNS, as well as in a DMS patient from a consanguineous family.

On the contrary, two amino acid substitutions, E237Q and A242V, were also found in healthy control patients. The E237Q substitution was identified in 2 sporadic cases and in 1 control individual (0.3%). This overall frequency in the healthy population is less than 1%, thereby not fulfilling the definition of an allelic polymorphism. Heterozygous A242V substitutions were sequenced in 7 patients: 2 with AR SRNS, 4 with sporadic SRNS, and 1 with DMS. It was also detected in 2 out of 320 control chromosomes (0.6%). Because most patients and both controls were of African origin, we expanded the control cohort to 75 individuals (150 chromosomes) of African descent and detected 6 individuals heterozygous for the A242V variant, demonstrating that it represents a polymorphism in the African population with an allele frequency of 4%.

In addition, the R229Q polymorphism was identified in 12 out of 320 healthy control chromosomes, defining a frequency of 3.75%, but never in the homozygous state in any control individual. This variant has recently been described by Tsukagushi et al [20] as a non-neutral polymorphism. In the present study, a R229Q substitution was detected in 3 families with AR SRNS and in one patient with sporadic SRNS, in addition to a pathogenic mutation on the other allele. Furthermore, homozygous R229Q mutations were identified in 3 AR SRNS (2 of them being consanguineous) and in 2 sporadic cases. No additional *NPHS2* mutations were detected in these patients. In 13 patients (11 with sporadic SNRS and 2 with DMS), we identified a heterozygous R229Q polymorphism without any other sequence variation in *NPHS2*. In total, the screening of 263 unrelated nephrotic patients (526 alleles) revealed 17 with heterozygous R229Q exchange (3.23%) and 5 with homozygous R229Q exchange (1.90%), resulting in an overall allele frequency in the nephrotic cohort of 5.13% ($P = 0.35$ when compared with the frequency of 3.75% in healthy control individuals). In contrast to what was observed with pathogenic mutations, these variants and polymorphisms were equally distributed either in the N-terminus (P20L and A61V) or the C-terminus part of the protein (L172V, R229Q, E237Q, A242V).

Age at onset

Analysis of the age at discovery of NS was performed among patients with and without pathogenic *NPHS2*

mutations for all cases in whom such data was available. Patients with pathogenic mutations in homozygous or compound heterozygous states manifested earlier (41.2 ± 5.9 months, $N = 63$) than patients without two pathogenic mutations in *NPHS2* for AR SRNS patients (69.9 ± 11.5 months, $N = 55$; $P = 0.019$) and for sporadic cases (108.3 ± 9.4 months, $N = 158$; $P < 0.001$). Similar results were found when only patients developing NS before 15 years of age were analyzed (41.5 ± 6.1 months, $N = 60$ vs. 57.9 ± 7.5 months, $N = 53$; $P = 0.053$ for AR SRNS and vs. 56.4 ± 4.1 months, $N = 127$; $P = 0.002$ for sporadic cases). With respect to a possible genotype-phenotype correlation, some trends were apparent. Patients with frameshift or nonsense mutations in the homozygous or compound heterozygous state had an early mean age of onset (21.9 ± 5.6 months, $N = 13$), as was the case with homozygous R138Q patients (11.8 ± 3.2 months, $N = 15$). Interestingly, in 3 patients with homozygous R138Q mutations, the first clinical symptoms appeared at birth, resembling an intermediate phenotype between congenital and infantile NS. We therefore performed *NPHS1* mutation analysis and found a third (heterozygous) mutation in *NPHS1* (275–2 A>C acceptor splice-site mutation) in 1 patient. *NPHS1* and *NPHS2* analysis in the parents of this patient revealed a heterozygous R138Q-*NPHS2* mutation in both parents, but no mutations were identified in *NPHS1*, thereby reflecting a de novo mutation in the patient.

On the contrary, some missense mutations were associated with milder clinical course. In particular, the V180M and the R238S variants were identified in patients with a later onset of SRNS with a median age of 128.7 ± 12.4 months ($N = 7$). Also, patients with a heterozygous R229Q exchange in addition to one pathogenic *NPHS2* mutation and patients with just one heterozygous mutation or variant of unknown significance have a later onset in our study cohort (88.1 ± 12.6 months, $N = 7$ and 147.4 ± 50.0 months, $N = 11$, respectively).

NPHS2 mutations and recurrence of proteinuria after renal transplantation

In total, 32 patients with *NPHS2* pathogenic mutations, either in the homozygous or compound heterozygous state, underwent kidney transplantation. Among them, only one developed recurrence of proteinuria two years after its third renal transplantation. This patient belongs to a consanguineous family with 3 affected children, and harbors the R138X nonsense mutation. The first and second renal allografts were lost immediately due to acute rejection and acute allograft failure, respectively. Two years after the third allograft (with mother as donor), nephrotic-range proteinuria was detected with renal biopsy disclosing both FSGS and tacrolimus toxicity, but no evidence of rejection. Having shown previously that no *NPHS2* mRNA could be detected in this patient's native kidney [11], we assayed for serum antipodocin antibodies by indirect immunofluorescence on normal human kidney. However, no antipodocin antibodies were found. In addition, *NPHS2* mutation analysis in 25 patients with sporadic SRNS and a recurrence of proteinuria after renal transplantation revealed heterozygous variants in 3 patients, namely T326fsX345, P20L, and E237Q (vs. 2 heterozygous variants/mutations out of 49 transplanted patients with sporadic SRNS and no recurrence of proteinuria after transplantation). The patient with a heterozygous pathogenic T326fsX345 mutation developed proteinuria 18 days after transplantation, and had FSGS on biopsy one year later, ultimately losing the allograft after four years. The second patient with heterozygous P20L variant had a rapid reappearance of proteinuria and allograft rejection immediately after receiving the first transplant. Because renal biopsy showed thrombotic microangiopathy, the contribution of the proteinuric state to allograft loss is likely minimal. However, three months after receiving a second transplant, the patient once again developed massive proteinuria, requiring plasmapheresis in addition to immunosuppressive therapy, including tacrolimus. The patient bearing the E237Q substitution presented with proteinuria and NS 18 months after renal transplantation, which resolved after treatment with iv cyclosporine A and plasmapheresis. In addition, a fourth patient with heterozygous R229Q exchange developed FSGS and proteinuria 22 months after renal transplantation. In all patients, subsequent direct sequencing of the entire *NPHS2* coding region and adjacent exon-intron boundaries revealed no second mutation/variant.

DISCUSSION

Our study involving 338 individuals presenting with SRNS represents the largest cohort of patients published to date, and includes both familial autosomal-recessive and sporadic cases. We established a significant contribution of mutations of the *NPHS2* gene encoding podocin because more than half (36/62) of families with AR SRNS were compatible with linkage. Pathogenic variants were discovered in 25 of these families, but not in those in whom linkage was not established. We would, therefore, advocate the use of haplotype analysis, using *NPHS2* flanking polymorphic markers as an initial diagnostic tool in these families, followed by more rigorous mutation detection when linkage is compatible. The detection of ~40% of mutations in the families with autosomal-recessive inheritance demonstrates the preponderance of *NPHS2* mutations in familial SRNS and confirms genetic heterogeneity in SRNS. This is a finding highly reminiscent of autosomal-dominant FSGS [28], and emphasizes the need for identifying additional genes

involved in familial forms of SRNS. We have extended previously published analyses of sporadic cases of SRNS [17, 19] by analyzing 172 patients. These patients have a significantly later onset of NS than familial cases. We report the identification of pathogenic mutations in either the homozygous or compound heterozygous or heterozygous state in 10.5% of cases, in agreement with results reported in the two groups of pediatric patients [17, 19]. The lower mutation detection rate among sporadic cases of SRNS than familial cases, and the delayed onset in those in whom mutations where not found, point to complex inheritance patterns (as evidenced by the presence of NS in distant relatives in seven cases), and to potential gene-environment interactions in patients with sporadic NS.

Furthermore, we have shown that *NPHS2* mutations are not causative in patients with *WT1*-negative cases of DMS. We included 19 patients with DMS in this cohort based on previous observations that patients with *WT1* mutations can present either with FSGS (Frasier syndrome) or DMS (Denys-Drash syndrome) [6, 7]. Furthermore, *Nphs2*-deficient mice develop renal failure secondary to DMS [29]. Our data, however, do not support a pathogenic role for podocin in this subset of patients.

We have identified 32 *NPHS2* variants, 26 of which are clearly pathogenic mutations on the basis of deleterious effects on gene expression or predicted structural alteration of the protein. Several of these mutations were identified in unrelated families. The majority of these are missense mutations (15/26), but also include frameshifting (7/26), in-frame deletion (1/26), nonsense (2/26), and splice-site (1/26) mutations. Of these, we here report 13 novel mutations in addition to four nucleotide substitutions of unclear significance based on a conservative change in the amino acid and the lack of residue conservation among stomatin family members. We have also identified the previously reported A242V variant [19] as a polymorphism enriched among patients of African origin. In addition, we sequenced the R229Q polymorphism with an overall frequency of 5.12% among our cohort of nephrotic patients.

The mutations are distributed throughout the entire gene, most affecting the C-terminal cytosolic domain. Interestingly, 21/26 variants affect the region of homology with the stomatin protein family [11]. The accumulation of mutations in this region correlates with the important functional roles ascribed to this domain, namely homo-oligomerization and interaction with other slit diaphragm proteins like nephrin, CD2AP, and the NEPH family [14, 15, 30]. Interestingly, genome database analyses point to a novel predicted gene (FLJ32940) expressed in human testis that partly overlaps with exon 6 of the *NPHS2* gene, but is oriented in the opposite direction. Fifteen nucleotides are shared by both genes, but no *NPHS2* mutation/variant identified so far affects any of the shared nucleotides. Were such mutations to occur, as would be the case with complete *NPHS2* deletions, patients might present with a different phenotype.

The nature of the mutation correlates to some extent with the age of onset of nephrotic syndrome. Patients with frameshifting and protein-truncating mutations present earlier with NS. Among missense variants, severity of the disease appears to be determined by the impact of the amino acid substitution on specific functional domains and on the intracellular trafficking of podocin. Our recent functional studies of the intracellular trafficking of several podocin mutants demonstrated that certain missense variants manage to reach the plasma membrane (V180M and R238S), while others, including R138Q, are retained in the endoplasmic reticulum (ER) [31]. Huber et al have similarly shown that the R138Q mutation, while retaining the ability to homo-oligomerize, is retained in the ER and fails to recruit nephrin to lipid rafts [16]. The R138Q mutation, which accounts for 32% of mutant alleles in our cohort, predicts mean onset of proteinuria before the first year of life when present in the homozygous state. In contrast, patients with the V180M and R238S mutations have a mean onset of NS much later than patients with the homozygous R138Q mutations (128 vs. 12 months). It will be interesting to see whether pharmacologic approaches that ameliorate the mistrafficking of the missense podocin variants will allow them to function properly, as has recently been shown for the ΔF508 CFTR protein [32].

With the enlargement of our cohort, we excluded the A242V substitution as a true mutation, as previously reported [19]. We sequenced this variant mostly in the African population, with an allele frequency of 4%. Additional studies will be required to determine whether its presence imparts an increased susceptibility to proteinuric renal disease. Likewise, we found the E237Q variant in two patients and in one control in our large cohort, therein, highlighting the difficulty of discriminating true mutations from rare polymorphisms. An equally difficult task is to ascribe pathogenicity to three missense variants seen in the heterozygous state, including the P20L substitution, which we identified in two cases (as the sole variant in one and in conjunction with a homozygous R168H mutation in another). This variant affects a nonconserved residue and in our functional analysis reaches the plasma membrane, while the R168H protein does not [31].

The identification of two pathogenic *NPHS2* alleles in SRNS patients predicts a more severe outcome characterized by earlier onset of disease. This underscores the crucial role of podocin in the maintenance of the filtration barrier. Among cases where only a single mutation/variant is detected, NS tends to occur later in life. Caridi et al reported heterozygous variants in patients with frequently relapsing NS [19], while we found one

such variant (A61V) in a patient with DMS. One possibility is that the existence of one hypomorphic podocin allele predisposes individuals to glomerular dysfunction in the presence of additional renal insults, as has been recently suggested by Kim et al regarding *CD2AP* haploinsufficiency [10]. We have observed the spontaneous development of proteinuria in some aged *Nphs2* heterozygous knock-out mice (C. Antignac, unpublished data). Alternatively, these podocin variants may act as modifiers in the setting of lesions in yet undefined genetic loci. Our own studies of *Nphs2* null mice have revealed a critical role for genetic modifiers evidenced by disparate rates of progression of renal failure and survival based on the underlying mouse strain [29].

Less severe phenotypes have also been noted in familial cases of FSGS involving a pathogenic mutation on one allele and the common polymorphism R229Q on the other allele [20]. Likewise, we detected seven patients belonging to four families with similar genotypes and a later age of onset of nephrotic syndrome. In addition, we found homozygous R229Q variant in five patients belonging to three unrelated families and in two sporadic cases.

Interestingly, despite a polymorphism frequency of 0.031, the homozygous R229Q variant has not been identified so far in control subjects. Although it has been shown in vitro to encode an altered protein with decreased binding affinity to nephrin [20], it remains difficult to imagine that the R229Q substitution alone, even in the homozygous state, might be responsible for a severe disease. It is likely that additional sequence mutations in *NPHS2* regulatory regions or introns, or variants in other genes, might explain the phenotype in these patients. It is, however, noteworthy that we found a higher frequency, though not significant, of the R229Q variant among nephrotic patients (5.13% vs. 3.75% in control patients), confirming previous findings of Tsukagushi et al [20]. Studies involving greater numbers of patients will be needed to determine the significance of this finding.

The severity of SRNS might also be modulated by the presence of additional mutations in genes encoding proteins interacting with podocin. *NPHS2* mutations associated with *NPHS1* variants have already been reported [19, 22], and we here describe a patient with very early onset SRNS in whom a heterozygous de novo splice mutation in *NPHS1* and a homozygous *NPHS2* R138Q mutation were detected. This is reminiscent of the triallelic inheritance found in some cases of Bardet-Biedl syndrome (BBS) [33, 34].

Contrary to previously published data by Bertelli et al [21], and as would be expected with the resulting structural alteration of the glomerular filter resulting from the podocin gene defect, we have found that recurrent disease after renal transplantation is very unlikely in patients with two *NPHS2* mutations. Out of 32 transplanted patients with two pathogenic *NPHS2* mutations, we observed only one patient, bearing the R138X mutation in the homozygous state, with recurrent FSGS and a delayed onset. This rate (1/32) is far below the current rate of recurrence of 25% in FSGS [1]. Actually, Bertelli et al [21] detected only two cases with recurrence of proteinuria after transplantation in patients with *NPHS2*-R138Q homozygous mutations, and in both cases, the patients had a mild episode with a favorable outcome. The mechanisms underlying these findings are not well defined. Pattraka et al recently described the presence of antiglomerular and antinephrin antibodies in the sera of transplanted patients with CNF who suffered from recurrent NS [35]. Although the development of antibodies directed against podocin is an attractive explanation for the recurrence of FSGS in our familial case of SRNS, our data and Bertelli's findings [21] do not support this. However, in agreement with the findings of Bertelli et al, we also found that 3 out of 5 sporadic SRNS patients bearing heterozygous mutations who were transplanted developed recurrence [21]. In these patients, the theory of antipodocin antibodies seems rather unlikely because podocin expression will not be completely abolished. The development of SRNS when it recurs early after transplantation is commonly regarded as arising from one or more suggested circulating permeability factor(s) that alter renal permeability, leading to proteinuria [2, 3]. As long as these circulating plasma factors remain unidentified, no clear conclusions can be drawn from genetic and clinical studies. Nevertheless, it can be suggested that *NPHS2* mutations/variants may play a role in modulating the progression of renal disease before transplantation.

CONCLUSION

The present study shows that the phenotypic spectrum of patients affected by two clearly pathogenic *NPHS2* mutations is restricted to severe early-onset SRNS and lack of recurrence of proteinuria after renal transplantation. This emphasizes the importance of *NPHS2* mutation screening in children with early-onset SRNS to provide accurate genetic counseling and clinical prognostication. However, heterozygous *NPHS2* mutations, sequence variants, and polymorphisms may play a role in atypical cases of SRNS with a later onset, mild clinical course, and recurring disease after renal transplantation. It can be expected that further genetic studies on families with *NPHS2*-negative SRNS will lead to the identification of new causative genes for SRNS.

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