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Nephrin mutations cause childhood- and adult-onset focal segmental glomerulosclerosis

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Mutations in the *NPHS1* gene cause congenital nephrotic syndrome of the Finnish type presenting before the first 3 months of life. Recently, *NPHS1* mutations have also been identified in childhood-onset steroid-resistant nephrotic syndrome and milder courses of disease, but their role in adults with focal segmental glomerulosclerosis remains unknown. Here we developed an *in silico* scoring matrix to evaluate the pathogenicity of amino-acid substitutions using the biophysical and biochemical difference between wild-type and mutant amino acid, the evolutionary conservation of the amino-acid residue in orthologs, and defined domains, with the addition of contextual information. Mutation analysis was performed in 97 patients from 89 unrelated families, of which 52 presented with steroid-resistant nephrotic syndrome after 18 years of age. Compound heterozygous or homozygous *NPHS1* mutations were identified in five familial and seven sporadic cases, including one patient 27 years old at onset of the disease. Substitutions were classified as 'severe' or 'mild' using this *in silico* approach. Our results suggest an earlier onset of the disease in patients with two 'severe' mutations compared to patients with at least one 'mild' mutation. The finding of mutations in a patient with adult-onset focal segmental glomerulosclerosis indicates that *NPHS1* analysis could be considered in patients with later onset of the disease.

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Idiopathic nephrotic syndrome (NS) represents a heterogeneous group of glomerular disorders occurring mainly in children. It is generally divided into steroid sensitive (SSNS) and steroid resistant (SRNS), depending on the patient's response to steroid therapy. Over the past decade, mutations in genes encoding podocyte proteins have been identified in several forms of hereditary NS.[1–6]

Mutations in the *NPHS1* gene are responsible for congenital NS of the Finnish type (CNF), which is an autosomal recessive disorder characterized by massive proteinuria often starting *in utero.*[7] Kidney biopsy shows irregular microcystic dilatation of proximal tubules[8] and the disease used to lead to death in the neonatal period, but nowadays it can be treated by dialysis and nutritional support, followed by renal transplantation in early childhood.[9] The human *NPHS1* gene is located at the long arm of chromosome 19, 19q13.1, and contains 29 exons.[10] The protein product, termed 'nephrin,' is a putative member of the immunoglobulin-like superfamily.[11,12]

Mutations in the *NPHS2* gene were initially described in early onset SRNS.[2] However, some cases with late onset disease have been described by Tsukaguchi *et al.*[13] and Machuca *et al.*[14] Variants in both *NPHS1* and *NPHS2* genes have been reported to occur together in a few number of families with congenital focal segmental glomerulosclerosis (FSGS)[15] or CNF,[16] suggesting the presence of 'oligogenicity' in this disorder.

At present, more than 90 mutations in *NPHS1* have been identified. These mutations are scattered along the *NPHS1* gene, most of them being private mutations.[15–20] The high percentage of *NPHS1* missense mutations represents a diagnostic challenge as in some cases it is difficult to differentiate between disease-causing variant and a neutral one. *In silico* approaches have recently been developed for the evaluation of amino-acid substitutions in several human disease genes.[21–24] These approaches take into consideration a combination of a multi-sequence alignment (MSA) of orthologous proteins and a measure of the chemical difference between the amino acids observed at the mutation point. These factors were used as *in silico* predictors in a scoring matrix for the evaluation of missense substitutions in the *NPHS1* gene.

Classically, *NPHS1* mutations have been observed in children presenting with NS within days after birth and before 3 months of life.[25] Recently, Philippe *et al.*[26] identified *NPHS1* mutations in children with later onset SRNS (between 5 months and 8 years). However, the role of *NPHS1* in adults with FSGS remains unknown. Our aim was to study if *NPHS1* mutations could be responsible not only for congenital-onset and childhood-onset but also for adult-onset FSGS.

RESULTS

Mutation analysis

NPHS1 mutation screening was performed in 97 patients from 89 families by direct DNA sequencing. Homozygous or compound heterozygous *NPHS1* substitutions were identified in 12 cases, of whom 1 was familial with two affected siblings, 4 were only children of consanguineous parents, and 7 were sporadic cases (Table 1). Two of these twelve cases carried additional variants in the *NPHS2* gene. In one CNF case (patient 19) we found the p.R229Q *NPHS2* variant in heterozygous state with two *NPHS1* mutations. Moreover, two compound heterozygous *NPHS2* variants (p.P20L and p.E237Q) in conjunction with a homozygous *NPHS1* mutation were detected in one patient with FSGS congenital NS (patient 41; Table 2).

In addition, only a single pathogenic *NPHS1* mutation was identified in two patients with sporadic SRNS, one of them (patient 88) carrying also the p.R408Q *NPHS1* neutral variant in heterozygosity (Table 2). Moreover, we identified two siblings with only one *NPHS1* mutation and the p.P20L variant in the *NPHS2* gene (family 21).

NPHS1 variants of unknown significance (p.N188I and p.P264R) were heterozygous in two cases (Table 2) and four patients carried a single new highly neutral *NPHS1* variant (p.Q259E in one case, p.L392P in three cases). Finally, the p.R408Q neutral variant was found in seven cases without any other variant in compound heterozygosity.

On the other hand, causative mutations in either *NPHS2* or *WT1* gene were found in eight cases (unpublished results), bearing no *NPHS1* variants. These patients were also screened for *TRPC6*, *ACTN4*, and *CD2AP* and no mutations were found.

The detection rate of *NPHS1* mutations for familial cases was 38% (5 of 13) and 10% (7 of 76) for sporadic cases. Patients with only one *NPHS1* mutation were not included to calculate this mutation detection rates (Table 1). If we discard patients clinically diagnosed as CNF, mutations were identified in 27% (3 of 11) of the familial cases and 6% (4 of 73) of the sporadic cases studied in the present paper. The frequency of *NPHS1* mutations in adults was 2% (1 of 52) but seven times higher in children (14%, 3 of 22).

Of the 25 *NPHS1* substitutions hereby detected, 72% (18 of 25) were missense. A large majority of these missense

Table 1 | Overview of genotypic and phenotypic data

	Familial SRNS	Sporadic SRNS	Congenital onset	Early childhood onset	Late childhood onset	Adolescent onset	Adult onset
No. of patients (families) studied	21 (13)	76 (76)	11 (10)	21 (16)	8 (6)	5 (5)	52 (52)
No. of patients (families) with two *NPHS1* mutations	6 (5)	7 (7)	8 (8)	3 (2)	1 (1)		1 (1)
Mutation detection rate	38.5%	10%	80%	13%	17%		2%
No. of patients (families) with one *NPHS1* mutation	2 (1)	2 (2)		2 (1)	2 (2)		
No. of patients (families) with one *NPHS1* variant of unknown effect		2 (2)					2 (2)
Age at onset in patients with 2 'severe' *NPHS1* mutations	1.1 ± 1.2 months; n=8 patients						
Age at onset in patients with 1 'mild'/1 'severe' or 2 'mild' *NPHS1* mutations	100 ± 130 months; n=5 patients						

SRNS, steroid-resistant nephrotic syndrome.
When 2 members of a single family presented an age at onset of the disease that fell between two different categories, we included both in the category of the patient presenting the earlier age at onset of NS.

Table 2 | Clinical data of patients with SRNS and *NPHS1* substitutions

Patient	Gender	Age of onset NS (months)	Renal biopsy	Therapy	Evolution	Tx/ Recurrence	*NPHS1* mutation 1 (MG)[a]	*NPHS1* mutation 2 (MG)[a]	*NPHS2* variants
Patients with CNF[b]									
18	M	0.6	CNF	—	ESRD at 4 years	Yes/No	c.1701C>A p.C567X (A)	c.2417C>G p.A806D (B)	Not identified
19	F	0.2	CNF	—	ESRD at 1 year	Yes/2 Tx[c]	c.1701C>A p.C567X (A)	c.3343G>T p.E1115X (A)	c.686G>A p.R229Q
20	M	0.2	CNF	—	ESRD at 1 year	Yes/2 Tx[c]	c.1701C>A p.C567X (A)	c.1868G>T p.C623F (B)	Not identified
38[d]	F	1	Not performed	—	ESRD at 1 year	No	c.2540_43del p.T847fsX903 (A)	c.2540_43del p.T847fsX903 (A)	Not identified
256[d]	M	1	Not performed	—	ESRD at 3 months	No	c.1379G>A p.R460Q (I)[e]	c.1379G>A p.R460Q (I)[e]	Not identified
Patients with congenital FSGS[b]									
40[d]	M	3	FSGS	Cs	ESRD at 2 years	Yes/2 Tx[c]	c.2143G>C p.G715R (B)	c.2143G>C p.G715R (B)	Not identified
41[d]	M	3	FSGS	Cs	Normal Cr at 3 years	No	c.1538T>C p.L513P (B)	c.1538T>C p.L513P (B)	c.59C>T p.P20 L+ c.709G>C p.E237Q
177	F	0	DMS	—	CKD stage IV at 2 years	No	c.139delG p.A47fsX127 (A)	c.3478C>T p.R1160X (A)	Not identified
Patients with childhood FSGS[b]									
79-1[f]	F	72	FSGS	Cs, CsA, MMF	Normal Cr at 15 years	No	c.1099 C>T p.R367C (B)	c.361G>A p.E121K (I)	Not identified
79-2[f]	M	12	FSGS*	Cs, CsA	Normal Cr at 7 years	No	c.1099 C>T p.R367C (B)	c.361G>A p.E121K (I)	Not identified
182	F	84	FSGS	Cs, CsA, CP	Normal Cr at 11 years	No	c.1379G>A p.R460Q (I)[e]	c.2928G>T p.R976S (B)[g]	Not identified
198	F	8	FSGS	Cs, CsA	Normal Cr at 2 years	No	c.791C>G p.P264R (C)	c.2026C>T p.P676S (I)	Not identified
Patient with adulthood FSGS[b]									
140	F	324	FSGS*	Cs, CP, Tacro[h]	Normal Cr at 29 years	No	c.2479C>A p.R827X (A)	c.2928G>T p.R976S(B)[g]/ c.2971G>C p.V991 L (NV)[i]	Not identified
Patients with one NPHS1 mutation									
21-1[f]	F	24	FSGS	Cs, CsA, MMF	Normal Cr at 8 years	No	c.3250_3251insG p.V1084fsX1095 (A)	Not identified	c.59C>T p.P20 L
21-2[f]	M	24	FSGS	Cs, CsA, MMF	Normal Cr at 6 years	No	c.3250_3251insG p.V1084fsX1095 (A)	Not identified	c.59C>T p.P20 L
85	M	115	FSGS	Cs, CsA, CP	ESRD at 12 years	Yes/Yes	c.3250_3251insG p.V1084fsX1095 (A)	Not identified	Not identified
88	F	132	FSGS*	Cs, CsA, CP	ESRD at 14 years	Yes/Yes	c.1610C>T p.T537 M (B)	c.1223G>A p.R408Q (P)	Not identified
Patients with one NPHS1 variant of unknown effect									
122	M	324	FSGS	Cs, CsA	ESRD at 28 years	Yes/Yes	c.563A>T p.N188I (I)	Not identified	Not identified
189	F	348	FSGS	Cs, CsA	ESRD at 34 years	Yes/No[c]	c.791C>G p.P264R (C)	Not identified	Not identified

CKD, chronic kidney disease; CP, cyclophosphamide; Cr, creatinine; Cs, corticosteroids; CsA, cyclosporin A; DMS, diffuse mesangial sclerosis; ESRD, end-stage renal disease; F, female; FSGS, focal segmental glomerulosclerosis; FSGS*, mesangioproliferative lesions with FSGS; M, male; MMF, mophetil micophenolate; Tacro, tacrolimus; Tx, kidney transplantation.

[a]*NPHS1* mutations, defined in this table as substitutions detected in the *NPHS1* gene; MG, mutation group (classification of *NPHS1* substitutions according to Table 3).

[b]These patients presented with 2 *NPHS1* mutations in homozygous or heterozygous state.

[c]These patients presented with chronic rejection nephropathy.

[d]Only child of consanguineous parents.

[e]This mutation has previously been described in patients with a severe phenotype.[25]

[f]Siblings with the same parents.

[g]This mutation has previously been described in patients with a mild phenotype.[26]

[h]This patient responded partially to CP treatment and she is now treated with angiotensin-converting enzyme inhibitor and Tacro.

[i]This patient has 3 heterozygous missense substitutions, but the p.V991L was considered a neutral variant.

changes were within the immunoglobulin motifs of the extracellular domain. As shown in Table 3, nine variants were novel (36%), consisting of seven missense, one nonsense, and one frameshift mutation. Although most mutations in this gene are private, we identified one mutation (p.C567X) in three out of five nonrelated patients with CNF (60%). Moreover, the p.R408Q neutral variant has an allele frequency of 4.5% (8 of 178 alleles) and the p.L392P neutral variant of 2% in our population with SRNS. Four more variants were present in at least more than one patient: p.P264R (exon 7), p.R460Q (exon 11), p.R976S (exon 22), and p.V1084fsX1095 (exon 24). No other hot spot was found in the *NPHS1* gene in the present study.

Classification of substitutions

Of the seven new missense substitutions, p.L513P, p.T537M, and p.G715R were classified as highly likely pathogenic mutations (mutation group (MG) = B) by our *in silico* scoring system analysis, p.E121K and p.P676S were classified as variants of unknown pathogenicity (MG = I), and finally, p.Q259E and p.L392P were classified as highly neutral variants (MG = NV) (Table 3). It is noteworthy that we identified three different amino-acid changes in heterozygous state in one patient (patient 140). One of them was a stop codon (p.R827X) in exon 18 and the remaining ones (p.R976S and p.V991L) were missense substitutions located in the fibronectin type III domain. Our scoring system analysis classified the p.V991L substitution as neutral variant. All new nonsense and frameshift mutations were predicted to result in a truncated protein.

The p.R976S variant (MG = B) should be considered a 'severe' mutation (see Materials and Methods) but it had previously been described in patients with a mild phenotype[15,26] and therefore was considered as 'mild' mutation. Even though the p.R460Q variant was predicted to be a variant of unknown effect (MG = I) by our *in silico* scoring system, it was considered a severe mutation because it had previously been identified in cases of CNF.[18,25,27,28]

Genotype–phenotype correlations

The mean age at onset of the disease in patients with congenital NS and compound heterozygous or homozygous *NPHS1* mutations was 1.1 month (from birth to 3 months) (Table 1). This group includes five patients with CNF, one with diffuse mesangial sclerosis, and two with congenital FSGS and SRNS (they did not receive immunosuppressant treatment). Among them, five patients had developed end-stage renal disease (ESRD) with a time interval of 1.7 years (range 1–4 years) from the onset of the disease to the development of ESRD. Although no disease recurrence was observed in four patients who received renal allograft, two of them developed chronic rejection nephropathy the first time they were transplanted. Moreover, patient 40 developed chronic rejection nephropathy both times he was transplanted (Table 2). Seven of the eight patients carried two *NPHS1* mutations classified as MG = A or MG = B (severe mutations). Patient 256 was homozygous for the p.R460Q mutation (MG = I).

The mean age at onset of disease in patients with childhood-onset NS and two *NPHS1* variants was 3.6 years (from 8 months to 7 years). Renal biopsy revealed mesangioproliferative lesions with FSGS in one patient and FSGS in three patients. These patients were resistant to corticosteroids as well as immunosuppressant drugs. At the end of follow-up, none of these patients had reached ESRD. All of them were compound heterozygous for at least one mutation classified as MG = C or MG = I (mild mutations).

The adult patient was diagnosed at 27 years of age and after 2 years of follow-up her renal function remains unimpaired. Renal biopsy of this patient showed FSGS and she responded partially to immunosuppressive treatment and angiotensin-converting enzyme inhibitor (proteinuria decreased to 3 g/24 h). In this patient, we detected two mutations classified as MG = A (p.R827X) and MG = B (p.R976S), and one neutral variant (p.V991L). We confirmed that p.R827X is from maternal and p.R976S from paternal origin.

The mean age at onset of NS in patients with one pathogenic *NPHS1* mutation identified was 6 years (from 2 to 11 years of age). The entire group of patients was resistant to corticosteroids as well as immunosuppressive treatment. At the end of follow-up, two patients had reached ESRD at 12 and 14 years of age. Two of them were transplanted and developed recurrence of proteinuria after kidney transplantation (Table 2). Finally, two patients with one variant of unknown significance developed SRNS in their adulthood and both reached ESRD. One of them developed recurrence of proteinuria after kidney transplantation.

DISCUSSION

NPHS1 mutations have been considered to cause a life-threatening disorder such as CNF for a long time. Although it is strictly true, Philippe *et al.*[26] have recently shown that patients carrying at least one 'mild' mutation have a much less severe phenotype than CNF. These results prompted us to look for mutations in this gene in an adult cohort of FSGS patients. The finding of a patient carrying two mutations in *NPHS1* and developing SRNS at 27 years of age shows that *NPHS1* mutations cause a renal disease that ranges from CNF to SRNS in childhood or adulthood. Similarly, it has been reported that congenital NS, previously only attributed to *NPHS1* mutations, is frequently caused by *NPHS2* mutations;[15,25] furthermore, *NPHS2* mutations have been found in adults with FSGS[13,29,30] and we have recently detected a child with FSGS and a *TRPC6* mutation,[31] although mutations in this gene had been described only in adults. Moreover, not only different genes are involved in SRNS but a combination of variants in different genes within the same individual may account for some cases of SRNS.[15,16,25,32] In the present study, we describe three cases with substitutions in both *NPHS1* and *NPHS2* genes (Table 2): one case (family 21) with one *NPHS1* mutation and one heterozygous *NPHS2*

Table 3 | Classification of *NPHS1* substitutions

NPHS1 substitutions	Exon	Previous description	GD[a]	GV[b]	GD/GV matrix score[c]	GDev[d]	Defined domain (degree of conservation)[e]	Splicing prediction[f]	Control chromo-somes	Described in SNP database	Polyphen prediction[g]	SIFT predicted tolerated[h]	VS[i]	MG[j]
Positive controls														
Protein is retained in endoplasmic reticulum: 'severe mutation'[26]														
R367C	9	[15,17,18]	180	85	+4	127 (0)	Ig 4 (HC) (+4)	Not predicted (0)	0/60[17] (+2)	No (+1)	2.16 (probably damaging) (+2)	No (+2)	15	B
C623F	14	[15–18,25]	205	0	+8	205 (+2)	Ig 6 NHC) (+2)	Not predicted (0)	0/60[17] (+2)	No (+1)	3.30 (probably damaging) (+2)	No (+2)	19	B
A806D	18	[17,18]	126	64	+3	124 (+2)	Ig 7 (NHC) (+2)	Not predicted (0)	0/60[17] (+2)	No (+1)	1.85 (possibly damaging) (+1)	No (+2)	13	B
L832P*	18	[26]	98	0	+6	98 (+2)	Ig 7 (NHC) (+2)	Not predicted (0)	0/182[26] (+2)	No (+1)	2.20 (probably damaging) (+2)	No (+2)	17	B
Protein traffics normally in the cell: 'mild mutation'[26]														
L96V*	3	[26]	32	0	+2	32 (+2)	Ig 1 (NHC) (+2)	Not predicted (0)	0/188[26] (+2)	No (+1)	1.34 (possibly benign) (−1)	No (+2)	10	C
A107T*	3	[26]	58	60	−2	30 (+1)	Ig 1 (NHC) (+2)	Not predicted (0)	0/188[26] (+2)	No (+1)	1.50 (possibly benign) (−1)	No (+2)	5	C
R460Q	11	[15,16,18,25,26]	43	139	−2	12 (+1)	Ig 5 (C) (+3)	Not predicted (0)	0/190[26] (+2)	No (+1)	1.40 (possibly benign) (−1)	Yes (−2)	2	I
P575Q*	13	[26]	76	58	−2	41 (+1)	Ig 6 (NHC) (+2)	Not predicted (0)	0/176[26] (+2)	No (+1)	1.80 (possibly damaging) (+1)	No (+2)	7	C
R976S	22	[26]	110	0	+6	110 (+2)	FTIII (C) (+3)	Normal AS:0.95 Mutant AS:0.79(0)	0/352[26] (+2)	No (+1)	2.20 (probably damaging) (+2)	No (+2)	18	B
Negative controls														
E117K	3	[17–19]	56	54	−2	22 (+1)	Ig 1 (NHC) (+2)	Not predicted (0)	22/60[17 k] (−2)	Yes (−1)	1.60 (possibly damaging) (+1)	Yes (−2)	−3	NV
R408Q	10	[17–19]	43	103	−2	17 (+1)	Ig 4 (HC) (+4)	Not predicted (0)	4/60[17] (−2)	Yes (−1)	1.75 (possibly damaging) (+1)	Yes (−2)	−1	NV
N1077S	24	[17–19]	46	241	−2	0 (−2)	No (0)	Normal AS:0.71 Novel AS:0.89(0)	2/60[17 k] (−2)	Yes (−1)	1.84 (possibly damaging) (+1)	No (+2)	−4	NV
No functional study performed														
N188I	5	[15]	149	145	0	10 (−2)	Ig 2(NHC) (+2)	Not predicted (0)	0/362[15] (+2)	No (+1)	1.80 (possibly damaging) (+1)	Yes (−2)	2	I
P264R	7	[15,18,32]	103	98	−2	46 (+1)	Ig 3(C) (+3)	Not predicted (0)	0/362[15] (+2)	Yes (−1)	1.93 (possibly damaging) (+1)	No (+2)	6	C
V991L	22	[16]	32	51	−2	0 (−2)	O (−4)	Not predicted (0)	0/196[16] (+2)	Yes (−1)	0.89 (benign) (−2)	Yes (−2)	−11	NV
Novel missense variants identified in our cohort														
E121K	3	Novel	56	124	−2	37 (+1)	Ig 1(NHC) (+2)	Not predicted (0)	0/200[l] (+2)	No (+1)	1.35 (possibly benign) (−1)	Yes (−2)	1	I
Q259E	7	Novel	29	82	−4	0 (−2)	O (−4)	Not predicted (0)	0/200[l] (+2)	No (+1)	0.25 (benign) (−2)	Yes (−2)	−11	NV
L392P	7	Novel	98	181	−2	0 (−2)	O (−4)	Not predicted (0)	2/200[l] (−2)	Yes (−1)	1.43 (possibly benign) (−1)	Yes (−2)	−14	NV
L513P	12	Novel	98	5	+6	95 (+2)	Ig 5(C) (+3)	Not predicted (0)	0/200[l] (+2)	No (+1)	2.07 (probably damaging) (+2)	No (+2)	18	B
T537M	12	Novel	81	0	+6	81 (+2)	Ig 5(C) (+3)	Not predicted (0)	0/200[l] (+2)	No (+1)	2.05 (probably damaging) (+2)	No (+2)	18	B
P676S	15	Novel	74	95	−2	34 (+1)	No (0)	Not predicted (0)	0/200[l] (+2)	No (+1)	1.7 (possibly damaging) (+1)	Yes (−2)	1	I
G715R	16	Novel	125	0	+6	125 (+2)	No (0)	Not predicted (0)	0/200[l] (+2)	No (+1)	2.3 (probably damaging) (+2)	No (+2)	15	B
Nonsense/frameshift substitutions														
A47fsX127	1	[20]	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	A
C567X	13	[18]	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	A
T847fsX903	19	Novel	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	A
R827X	18	[16,26]	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	A
V1084Xfs X1095	24	[10,20]	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	A

Table 3 continued on the following page

Table 3 | Continued

NPHS1 substitutions	Exon	Previous description	GD[a]	GV[b]	GD/GV matrix score[c]	GDev[d]	Defined domain (degree of conservation)[e]	Splicing prediction[f]	Control chromosomes	Described in SNP database	Polyphen prediction[g]	SIFT predicted tolerated[h]	VS[i]	MG[j]
E1115X	26	Novel	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	A
R1160X	27	15–18,20,25	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	A

NR, not required; SIFT, sort intolerant from tolerant algorithm.
*Substitutions not detected in our cohort of patients.
[a]GD (Grantham distance); score of chemical difference between the normal and mutated residue (high score, greater difference).
[b]GV (Grantham variation); score of chemical difference between 14 orthologs (ranging from orangutan to fruit fly) (0=completed conserved).
[c]GD/GV matrix score; lower matrix scores corresponded to low GD and high GV (conservative change and strong variation within the MSA), whereas higher matrix scores corresponded to high GD and low GV (nonconservative change and strong conservation within the multi-sequence alignment).
[d]GDev (Grantham deviation); score of chemical difference between the mutated residue and the range of variation between orthologs (GD similar to GDev, higher difference).
[e]Domain-containing residue: Ig-like C2 type domains (1–8), fibronectin type II (FTIII), Cter (1160–1241): binding to podocin. C, conserved (80–50%); domains; HC, highly conserved (>80%); NHC, not highly conserved (49–30%); No, not defined.
[f]Not predicted by Splice Site Prediction Neural Network. Score of the acceptor site (AS).
[g]Polyphen assessment; ratio Polyphen >2 (probably damaging), ratio Polyphen >1 (possibly damaging), ratio Polyphen <1 (benign).
[h]SIFT tolerated; not tolerated, tolerated.
[i]Variant score (VS).
[j]VS > 11 → mutation group (MG)=B; VS=5–10→MG=C; VS=0–4→MG=I; VS <−1→MG=NV; A, pathogenic; B, highly likely pathogenic; C, likely pathogenic; I, unknown pathogenicity; NV, neutral variant.
[k]These substitutions have been founded in control subjects in homozygous state.
[l]This report.

variant; one patient (patient 19) with two recessive *NPHS1* mutations and the p.R229Q *NPHS2* variant; and finally, one patient with ‘four-allelic hit’ (patient 41): one homozygous *NPHS1* mutation and two *NPHS2* variants. This group includes one family with early onset SRNS, one congenital FSGS, and one case of CNF. Contrary to these findings, Koziell *et al.*[15] reported substitutions in both *NPHS1* and *NPHS2* genes in cases with congenital FSGS. All patients reported in the literature and in the present study show two recessive mutations in one of these genes, which seem to be sufficient to explain their phenotype. Generally, the substitution identified in the other gene can be considered as a variant. Moreover, no significant clinical difference was observed between patients with CNF and with or without a third hit in one of these genes. In these three cases, our data do not suggest that patients with combined variants in *NPHS1* and *NPHS2* would result in phenotypic modification.

Missense mutations are the type most frequently found in this study (72%). As previously reported by Philippe *et al.*,[26] we tried to classify these substitutions as ‘severe’ or ‘mild’. We used previously reported *NPHS1* missense mutations and polymorphisms for which functional assays have been already carried out[26,33] to test the performance of our *in silico* scoring matrix for the *NPHS1* gene (Table 3). The resulting phenotype from missense mutations might depend on functional effects such as intracellular nephrin trafficking because some mutant proteins are retained in the endoplasmic reticulum.[26,33] According to these data, nonsense and frameshift mutations, which are predicted to result in a truncated protein, were considered severe as were missense mutations preventing nephrin from reaching the plasma membrane. These missense substitutions were classified by our *in silico* scoring system analysis as highly likely pathogenic mutations (MG = B). On the other hand, missense substitutions were designated as mild when functional analysis demonstrated partial maintenance of the protein function.[26,33] In our *in silico* system, these missense substitutions were classified as likely mutations (MG = C) or variants of unknown effect (MG = I), whereas p.E117K and p.N1077S, which have been found as homozygous changes in healthy controls,[17,18] were classified as neutral variants (MG = NV) (Table 3). The new nonsense, frameshift and p.L513P, p.T537M, and p.G715R missense mutations found in our study cohort were classified by our *in silico* scoring system as MG = A or MG = B and were therefore classified as severe. The other two new missense variants (p.E121K and p.P676S) were classified as variants of unknown effect (MG = I) and were consequently classified as mild. The p.R408Q variant, which is found in ~6%[17,19] of healthy controls in heterozygosity, was classified by our scoring system as a neutral variant. Interestingly, the homozygous p.R408Q variant has not been identified so far in control subjects. Even though we did not find a significant higher frequency in our population of patients with SRNS (4.5%) and it has been shown *in vitro* to reach the cell membrane,[33] studies involving greater numbers of patients will be needed to determine the significance of this variant.

In spite of the strict method used to classify missense variants, there were some discrepancies between genetic, functional, and phenotypic data. For instance, although the p.R976S variant is predicted to be a highly likely pathogenic mutation (MG = B), it had previously been classified as a mild mutation by Philippe *et al.*[26] because this mutant nephrin maintains its capacity to traffic in the cell membrane. However, its capacity to homodimerize and heterodimerize with NEPH1 has not been tested.[26] Another example is the p.R460Q mutant, which is predicted to be a tolerated variant and has been shown to traffic normally in the cell membrane and to homodimerize and heterodimerize with NEPH1, however, it had previously been identified in homozygosity in cases of CNF,[18,25,27] suggesting that this mutation may

disrupt other structural or functional properties of nephrin.[34–36] Therefore, it seems that functional or *in silico* analysis is a good tool to differentiate between mutations and polymorphisms but is not an infallible method to differentiate between mild and severe missense mutations.

We have found recessive mutations in five familial and seven sporadic cases, of which eight had congenital-onset, three had childhood-onset, and finally, one presented with adult-onset FSGS. Our data suggest that patients with two *NPHS1* severe mutations seem to present congenital onset of the disease, whereas patients with at least one mild *NPHS1* mutation present an early or late childhood onset of the NS, as reported by Philippe *et al.*[26] On the basis of this hypothesis, we expected patients with two mild *NPHS1* mutations to debut with an adulthood FSGS. However, the two *NPHS1* mutations identified in the adult patient (patient 140) were one nonsense mutation (p.R827X) and the p.R979S missense mutation, which has been considered a mild mutation. Conversely, two probably mild mutations were identified in patient 198 (p.P264R and p.P676S) presenting with an early childhood onset of NS. Therefore, we did not find any apparent difference between being a carrier of two mild mutations or one mild and one severe mutation. Interestingly, exactly the same combination of *NPHS1* mutations found in the adult patient has also been identified in one case with an age at onset of NS at 9 months.[26] Thus, modifying genes and environmental factors may account for the late onset of disease and the preservation of renal function 2 years after the diagnosis. This patient was the sole adult patient with compound heterozygous *NPHS1* mutations, which means that it is very uncommon to find two mutations in the *NPHS1* gene in adult patients with FSGS.

This limited cohort of patients carrying *NPHS1* mutations showed no response to immunosuppressive therapy. Only the adult patient reduced her rate of proteinuria from nephrotic to non-nephrotic with immunosuppressive therapy and angiotensin-converting enzyme inhibitor. Although our patients with CNF and severe mutations did not respond to any therapy, it has been reported that some missense mutations lead to milder therapy response forms of CNF. Furthermore, no patient in this cohort with two mutations in the *NPHS1* gene showed recurrence of proteinuria after transplantation, which supports the theory that only patients with the Fin major mutation in homozygosity, which implies a total loss of the protein, develop antibodies able to attack the slit diaphragm.[37]

In conclusion, this is the first description of *NPHS1* mutations being the cause of FSGS in adults. This is a clear example of how much work is still needed to understand well the pathogenesis of SRNS and FSGS. Many more genes than those already known may be involved in this disease. Moreover, those already known may account for totally unexpected phenotypic expression, and interactions among them also remain to be elucidated in depth.

MATERIALS AND METHODS

Patients

Mutation screening was performed in a cohort of 97 Spanish familial and sporadic cases with SRNS. Among them, 21 patients belonging to 13 families were considered as familial cases, defined either as families in which two members of a single generation were affected ($N=8$) or families in which one affected child was the product of a consanguineous relationship ($N=5$). Seventy-six patients presented with sporadic SRNS (Table 1).

We classified our population, according to the age at onset of the disease (mean ± s.d.), as: congenital (<3 months; 1.2 ± 1 month; $N=10$, including five CNF cases), early childhood (>3 months to 5 years; 21.4 ± 15.4 months; $N=21$), late childhood (6–12 years; 98.8 ± 30.8 months; $N=8$), adolescent (13–18 years; 15.6 ± 1.7 years; $N=5$), and adult (>18 years; 33.3 ± 9.7 years; $N=52$).

Patients that were born prematurely, presented with a large placenta and severe proteinuria at birth or had proven renal biopsy were classified as CNF. The NS was resistant to corticosteroids in all cases. When additional treatments (cyclosporine A, cyclophosphamide, or mycophenolate) were attempted, 10 of 48 patients responded partially or completely. Renal biopsy was available in the entire group of 52 patients with adult –onset NS. Thirty-seven patients showed FSGS, one patient mesangial IgM, three patients minimal change NS, and eleven patients mesangioproliferative lesions with FSGS, defined by the Columbia FSGS classification system.[38]

In this work, we will refer to the number of patients studied when assessing clinical data and to the number of families studied when assessing genetic data, because affected siblings may follow a different clinical course but should bear the same mutations.

Mutation analysis

Peripheral blood samples were obtained after informed consent of patients or their parents. Genomic DNA was isolated from peripheral blood samples using the 'salting out' method.[39] Mutation analysis of *NPHS1* gene was carried out by direct sequencing using previously described primers[17] and using the Big Dye terminator method (Applied Biosystems, Foster City, CA, USA). In this cohort of patients, *NPHS2*, *WT1*, *TRPC6*, *ACTN4*, and *CD2AP* sequencing analysis was also performed as previously described.[5,28] Segregation of the detected variants was confirmed by direct sequencing of parental DNA samples when available.

Classification of substitutions

To classify missense substitutions as a deleterious/high-risk variants or as neutral/little clinical significance, we have developed an *in silico* scoring system based on previous reports,[21–24,40] taking into account seven types of data: (1) the biophysical and biochemical difference between the wild-type and the mutant amino acid (Grantham distance);[40] (2) the evolutionary conservation of the amino-acid residue in an MSA of ortholog nephrin proteins (Grantham variation).[21] Grantham variation was defined as the largest Grantham distance for a specific position within the MSA. The MSA was generated using ClustalW software (http://www.ebi.ac.uk/tools/clustalw2).[41] Sequences were obtained from NCBI or ENSEMBL (human, chimpanzee, orangutan, rhesus monkey, cow, dog, mouse, rabbit, gray opossum, xenopus, zebrafish, mosquito, fruit fly); (3) the distance between the mutant amino acid and the range of variation present at their position in MSA (Grantham deviation);[22] (4) evaluation of each variant for affecting splicing using the algorithm known as the Neural Network Splice Site

Prediction; (5) identification of the defined domains (immunoglobulin-like (1–8), fibronectin type III, and the region binding to podocin (encoding by *NPHS2* gene)) using UniProtKB/Swiss-Prot program and the published literature;[17] (6) inclusion of contextual information: population data such as previous description of the variant in databases (as derived from the Human Gene Mutation Database and the Single Nucleotide Polymorphism Database). When a missense variant was not previously described, we analyzed 200 normal chromosomes (matched by ethnicity and geography with the study cohort); and (7) evaluation of variants using 'sort intolerant from tolerant' (SIFT) (http://sift.jcvi.org)[42] and 'Polymorphism Phenotype' (Polyphen) (http://genetics.bwh.harvard.edu/pph)[43] programs.

We assigned points for each of these factors, the sum of which resulted in an overall variant score (VS) (as previously suggested by Rossetti *et al.*[23]). The overall VS was classified into four groups, specifically VS equal to or less than −1 (MG = NV, highly neutral variant), VS between 0 and 4 (MG = I, unknown effect variant), VS between 5 and 10 (MG = C, likely pathogenic), and VS higher than 11 (MG = B, highly likely pathogenic). Nonsense and frameshift mutations were classified as MG = A (pathogenic mutations).

The scoring matrix was tested using previously described and classified amino-acid substitutions for which functional studies had been performed as positive controls (pathogenic variants, positive training set) or negative controls (neutral variants/polymorphisms, negative training set) (Table 3). The values assigned to each specific factor are inspired by the scoring matrix developed for the *PKD1/2* genes[23] with some minor modifications following the testing and training of the scoring matrix to the *NPHS1* gene. The trained scoring matrix was then used to evaluate the actual amino-acid substitutions found in our study cohort.

We attempted to classify these mutations as either 'severe' or 'mild' on the basis of the suggestive data by Philippe *et al.*[26] Nephrin mutations were classified as severe when they were predicted to result in a truncated protein (MG = A) or as highly likely missense mutations (MG = B) by our scoring system analysis. On the other hand, missense mutations classified as MG = C or variants of unknown effect MG = I were designated as mild.

DISCLOSURE

All the authors declared no competing interests.

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Appendix

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