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Clinical and epidemiological assessment of steroid-resistant nephrotic syndrome associated with the *NPHS2* R229Q variant

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Mutations of *NPHS2*, encoding podocin, are the main cause of autosomal recessive steroid-resistant nephrotic syndrome (NS) presenting in childhood. Adult-onset steroid-resistant NS has been described in patients heterozygous for a pathogenic *NPHS2* mutation together with the p.R229Q variant. To determine the frequency and the phenotype of patients carrying the p.R229Q variant, we sequenced the complete coding region of *NPHS2* in 455 families (546 patients) non-responsive to immunosuppressive therapy or without relapse after transplantation. Among affected Europeans, the p.R229Q allele was significantly more frequent compared to control individuals. Thirty-six patients from 27 families (11 families from Europe and 14 from South America) were compound heterozygotes for the p.R229Q variant and one pathogenic mutation. These patients had significantly later onset of NS and end stage renal disease than patients with two pathogenic mutations. Among 119 patients diagnosed with NS presenting after 18 years of age, 18 patients were found to have one pathogenic mutation and p.R229Q, but none had two pathogenic mutations. Our study shows that compound heterozygosity for p.R229Q is associated with adult-onset steroid-resistant NS, mostly among patients of European and South American origin. Screening for the p.R229Q variant is recommended in these patients, along with further *NPHS2* mutation analysis in those carrying the variant.

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Idiopathic focal segmental glomerulosclerosis (FSGS) represents a heterogeneous clinical entity in terms of response to immunosuppressive therapy, progression to end-stage renal disease (ESRD), and recurrence after kidney transplantation. Approximately 10–20% of children and 40% of adults presenting with idiopathic nephrotic syndrome (NS) do not achieve sustained remission after steroid therapy; up to 60% of these cases reach ESRD within 10 years of disease onset.[1–4] Overall, recurrence of NS after transplantation is observed in 30% of patients.[5,6] A putative circulating factor disrupting the glomerular filtration barrier has been hypothesized as the etiology of cases with response to steroids and other immunosuppressive therapy, as well of those recurring after transplantation.[7,8] In the last few years, advances in molecular genetics of familial NS led to the discovery of molecules essential for the maintenance of podocyte slit diaphragm structure and function, including nephrin (*NPHS1*),[9] podocin (*NPHS2*),[10] α-actinin-4 (*ACTN4*),[11] CD2-associated protein (*CD2AP*),[12,13] transient receptor potential channel 6 (*TRPC6*), [14,15] and phospholipase C epsilon (*PLCE1*).[14,15] Podocin is a 383-amino-acid lipid-raft-associated protein localized at the slit diaphragm, where it is required for the structural organization and regulation of filtration function. Interactions with nephrin, NEPH1, CD2AP, and TRPC6 manage mechanosensation signaling, podocyte survival, cell polarity, and cytoskeletal organization.[16–19]

NPHS2 mutations were initially described in early-onset steroid-resistant nephrotic syndrome (SRNS).[10] Subsequent studies further defined the phenotype associated with mutations in this gene, revealing that patients usually develop NS from birth to 6 years of age, present mostly with FSGS, do not respond to immunosuppression, and reach ESRD before the end of the first decade of life.[20–23] However, cases with late-onset disease have been described by Tsukaguchi *et al.*,[24] who found mutations in one-third of families with

autosomal-recessive late-onset FSGS. In six of these nine families, affected individuals were compound heterozygous for a particular variant, p.R229Q, and one pathogenic mutation. The p.R229Q variant is the most frequently reported non-synonymous *NPHS2* variant in Caucasians.[25] It is more common in Europeans, in whom the observed frequency of heterozygotes ranges from 0.03 to 0.13.[20,21,24–27] The pathogenic role of this variant has been suggested from *in vitro* studies showing decreased nephrin binding to podocin p.R229Q. Additional evidence arguing for a pathogenic role of p.R229Q is the conservation of the arginine 229 residue in podocin orthologs and the fact that arginine to glutamine is a non-conservative amino-acid change.

To evaluate the epidemiological relevance, clinical features, and kidney disease progression in SRNS patients carrying the p.R229Q variant, we screened for podocin mutations in a worldwide cohort of patients with SRNS, focusing our analyses on juvenile and adult forms of SRNS.

RESULTS

Demographic and clinical characteristics

We screened for *NPHS2* mutations in a cohort of 546 patients from 455 families with SRNS, in whom we previously excluded those cases with a potential underlying immune disorder defined by remission after immunosuppressive therapy, late steroid resistance, or relapse after transplantation. Most of the families originated from Europe (55.2%), although others from the Middle East (11.9%), North Africa (11.4%), South America (Chile and Argentina; 10.3%), Asia, the Caribbean and Polynesia (7.5%), and Sub-Saharan Africa (3.3%) were included. Out of 546 patients, 286 corresponded to sporadic cases, whereas 235 patients from 144 families were classified as familial forms. No information was available to classify 25 patients. Overall, the age at onset of NS ranged from 0 to 73 years (median 6 years), ESRD was reached by 272 patients (median 13 years), and 148 patients were transplanted (Table 1).

In total, 119 (24.4%) patients developed NS after 18 years of age. Within this group, 81 were sporadic cases, 36 (from 25 families) were familial forms, whereas no information was available to classify the remaining two patients.

Pathogenic *NPHS2* mutations and p.R229Q allele distribution in SRNS patients

We identified 104 patients (from 65 families) carrying pathogenic *NPHS2* mutations in the homozygous or compound heterozygous state. In addition, 63 cases from 54 unrelated families were heterozygous for the p.R229Q variant, out of which 36 cases from 27 families carried the p.R229Q variant associated with one pathogenic *NPHS2* mutation. In the other 27 patients, p.R229Q was found in the single heterozygous state. We also detected eight cases from five families that were homozygous for p.R229Q (Figure 1).

To evaluate the putative pathogenic role of the p.R229Q allele among SRNS patients, we performed the subsequent analyses excluding those cases carrying two pathogenic *NPHS2* mutations as they had an obvious disease cause.

Table 1 | Clinical and histological features in patients with SRNS according to *NPHS2* mutation status

Clinical feature	(1) R229Q+1 mutation	(2) R229Q+R229Q	(3) 2 mutations	(4) 1 R229Q+1 wt	(5) 1 mutation+1 wt	(6) No mutations	Total
Age at nephrotic syndrome onset							
Cases with available information (total)	33 (36)	8 (8)	92 (104)	26 (27)	5 (5)	323 (366)	487 (546)
Mean ± s.d. (years)	17.3 ± 10.5	6.3 ± 3.8	3.0 ± 3.6	17.7 ± 16.1	12.3 ± 17.8	12.1 ± 12.3	11.0 ± 12.1
Median (range)	19 (0–39)	6.8 (0.6–10.7)	1.1 (0–13.7)	14.8 (0.9–73.5)	6.5 (1.3–43.7)	7.9 (0–56)	6 (0–73.5)
Age at ESRD							
Cases with available information (total)[a]	14 (17)	5 (5)	51 (52)	9 (9)	1 (1)	173 (188)	253 (272)
Mean ± s.d. (years)	26.4 ± 10.1	10.8 ± 4.4	8.6 ± 5.2	26.1 ± 18.9	—	18.8 ± 14.4	17.2 ± 13.8
Median (range)	27.9 (9.3–43.5)	11.2 (3.4–14.5)	8.0 (0–26)	25.4 (1.1–62)	—	15.0 (0.3–57)	13 (0–62)
Histology (last kidney biopsy)							
Number of cases with kidney biopsy	33	7	75	26	5	296	442
Minimal change disease, *n* (%)	1	2	18	4	2	59	86 (20)
FSGS, *n* (%)	32	4	39	19	3	204	301 (68)
Diffuse mesangial proliferation, *n* (%)	—	1	16	2	—	17	36 (8)
Other, *n* (%)	—	—	2	1	—	16	19 (4)

ESRD, end-stage renal disease; FSGS, focal segmental glomerulosclerosis; wt, wild-type.
Mutations comprise splice and truncating mutations as well as missense mutations that are known or predicted to be deleterious. Patients were grouped according to the genotype as follows: (1) patients carrying one pathogenic mutation and the p.R229Q variant on the other allele (36 cases from 27 families); (2) patients homozygous for p.R229Q (8 cases from 5 families); (3) patients with two pathogenic mutations (104 cases from 65 families); (4) patients carrying one wild-type allele and the p.R22Q variant (27 cases from 27 families); (5) patients carrying one pathogenic mutation and a wild-type allele (5 cases from 4 families); and (6) patients carrying two wild-type alleles (366 cases from 327 families).
[a]Number of individuals reaching ESRD.

Allele frequency calculations were performed considering only one affected case per family to avoid overestimations. We performed a stratified analysis to compare the p.R229Q allele frequency between SRNS patients and controls of similar ethnic background (Table 2). The difference in the p.R229Q allele distribution was highly significant among Europeans (cases 0.089 vs controls 0.026; $P = 1 \times 10^{-5}$). Similarly, the p.R229Q allele was more frequent among South American patients than among controls (0.17 vs 0.007; $P = 2 \times 10^{-6}$), whereas no difference was observed among individuals from Middle East or North Africa. Comparisons among other ethnic groups were not performed due to sample-size limitations.

Unexpectedly, we observed a high frequency (31/390 = 8%) of patients carrying pathogenic mutations in the heterozygous state (Table 3). Therefore, we evaluated the association between a pathogenic mutation in one allele and the p.R229Q variant in the other. The proportion of individuals carrying a single pathogenic *NPHS2* mutation was significantly higher in those who additionally had one p.R229Q allele than in those carrying a wild-type allele ($P = 3 \times 10^{-21}$). This finding strongly suggests that p.R229Q in the compound heterozygous state with a *NPHS2* mutation has a pathogenic role in SRNS.

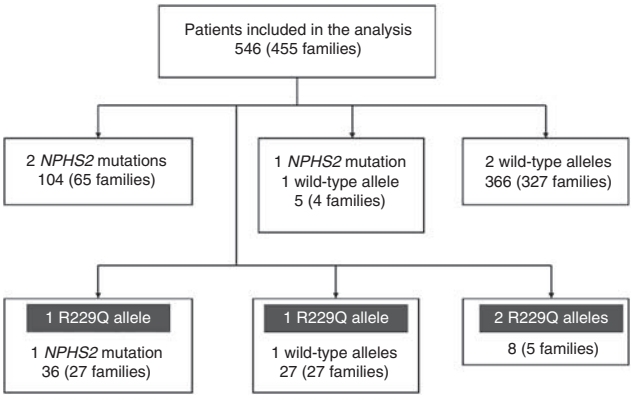

Figure 1 | SRNS patient distribution according to genotype. Flow diagram depicting patient classification according to genotype.

Among Europeans, the proportion of affected cases carrying p.R229Q in the homozygous state did not differ from controls (3/214 vs 0/308; $P = 0.07$). Moreover, four individuals from two families from the Middle East or North Africa were homozygous for the p.R229Q variant.

Patients carrying p.R229Q and one *NPHS2* mutation present frequently with juvenile and adult-onset SRNS

Thirty-six patients from 27 families were included in this group (21 familial and 15 sporadic cases). Mutations, clinical characteristics, and renal histopathology features are summarized in Table 4. Segregation analysis performed in all available family members showed that all patients were compound heterozygotes for p.R229Q and the pathogenic mutation. The pattern of inheritance was consistent with an autosomal-recessive disease with complete penetrance. Assessment of the clinical status of the parents and siblings of each patient confirmed the absence of glomerular disease in heterozygous carriers.

All except three cases in this group were compound heterozygous for missense mutations; p.A284V was found in 15 families, 13 of them from South America (Chile and Argentina) and 2 from Europe. We found three novel mutations: c.643C>T (p.Q215X), c.929A>C (p.E310A), and c.964C>G (p.R322G). Sequence alignment revealed that glutamic acid and arginine at positions 310 and 322, respectively, are highly conserved among podocin orthologs (data not shown). Substitutions for alanine at position 310 and glutamine at position 322 were predicted to be

Table 3 | Association of pathogenic *NPHS2* mutations with the p.R229Q or the wild-type allele

	Pathogenic mutations		
R229Q	1 mutation	No mutated allele	Total
1 or 2 R229Q alleles[a]	27	32	59
No R229Q allele	4	327	331
Total	31	359	390

Patients carrying two pathogenic mutations were excluded from the analysis. Calculations based on one affected case per family.
[a]Includes five cases homozygous for p.R229Q. Among SRNS patients, the proportion of subjects carrying one pathogenic mutation was much higher ($P=3 \times 10^{-21}$) when they also carried one R229Q allele (27/59=46%) than when they carried a wild-type allele (4/331=1%).

Table 2 | Genotypes and p.R229Q allele distribution in SRNS and controls based on one affected case per family

	SRNS					Controls				
Ethnic group	GG	GA	AA	Total	MAF	GG	GA	AA	Total	MAF
Europeans[a]	179	32	3	214	0.089	292	16	0	308	0.026
South Americans[a,b]	31	16	0	47	0.170	69	1	0	70	0.007
African-sub-Sahara and African-American	15	0	0	15	—	—	—	—	—	—
Northern Africa and Middle-East	75	3	2	80	0.045	88	7	0	95	0.037
Others and of unknown origin[c]	31	3	0	34	0.044	14	0	0	14	—

A, minor allele; MAF, minor allele frequency.
Frequencies were calculated using one affected case per family. In the group with SRNS we exclude patients carrying two pathogenic mutations (65 unrelated cases).
[a]Distribution among cases and controls reached statistical significance ($P<0.01$).
[b]All South American cases and controls were of Spanish descent. Others: includes French-Caribbean, Polynesian, and Asian population.
[c]Ethnic origin uncertain in two cases.

Table 4 | Ethnic origin, phenotype, and kidney histology in patients carrying p.R229Q in the compound heterozygous state

ID	Mutation	Ethnic origin	Disease onset (years)	NS onset (years)	Therapy/ effect	eGFR decline (ml/min/year)	Age at dialysis (years)	eGFR last visit (years)	Histology age (years)
1a	p.[R229Q]+ [A284V]	Europe	7	Yes (16)	CS-CyA-	32	20	—	MCD (12) FSGS (18)
2a	p.[R229Q]+ [A284V]	Europe	0	Yes (0.13)	CS-CyA ± (w/ACEI)	27.5	20	—	IGL (0) FSGS (13)
3a	p.[R229Q]+ [A284V]	South America	20	Yes (23)	CS-	11	—	97 (25)	FSGS (23)
3b	p.[R229Q]+ [A284V]	South America	19	Yes (21)	CS-	1	—	98 (23)	FSGS (21)
4a	p.[R229Q]+ [A284V]	South America	4	Yes (4)	CS-CyA-	Unknown	—	59 (12)	FSGS (5)
4b	p.[R229Q]+ [A284V]	South America	10	Yes (10)	CS-	17.5	10	—	FSGS (10)
5a	p.[R229Q]+ [A284V]	South America	7	Yes (7.5)	CS-CyA-Chl-	23.3	17	—	FSGS (8)
5b	p.[R229Q]+ [A284V]	South America	10	Yes (10)	No treatment	—	—	>80 (18)	FSGS (10)
6a	p.[R229Q]+ [A284V]	South America	10	Yes (20)	No treatment	8	30	—	FSGS (22)
6b	p.[R229Q]+ [A284V]	South America	4	Yes (21)	No treatment	3.25	33	—	FSGS (21)
7a	p.[R229Q]+ [A284V]	South America	24	Yes (24)	CS-	Unknown	—	Unknown	FSGS (24)
8a	p.[R229Q]+ [A284V]	South America	15	Yes (15)	CS-	Unknown	—	65 (15.5)	FSGS (15)
9a	p.[R229Q]+ [A284V]	South America	25	Yes (25)	CS-CyA-	29.7	28	—	FSGS (25)
10a	p.[R229Q]+ [A284V]	South America	15	Yes (15)	CS-CyA-	32.4	—	21 (19.5)	FSGS (15)
11a	p.[R229Q]+ [A284V]	South America	19	Yes (19)	CS-CyA-MMF-EDX-	6.7	—	91 (27.5)	FSGS (19)
12a	p.[R229Q]+ [A284V]	South America	15	Yes (15)	CS-CyA-EDX-	40.6	18	—	FSGS (15)
13a	p.[R229Q]+ [A284V]	South America	13	Yes (15)	CS-CyA-MMF-	7.1	26	—	FSGS (13)
14a	p.[R229Q]+ [A284V]	South America	16	Yes (16)	CS-MMF-	36	—	70 (16)	FSGS (16)
15a	p.[R229Q]+ [A284V]	South America	25	Yes (25)	CS-CyA-	8.2	—	19 (33.5)	FSGS (25)
15b	p.[R229Q]+ [A284V]	South America	28	Yes (28)	CS-CyA-	0	—	116 (33)	FSGS (28)
16a	p.[R229Q]+ [R285fx302X]	Europe	20	Yes (20)	No treatment	28	30	—	FSGS (26)
17a	p.[R229Q]+ [R285fx302X]	Europe	24	Yes (24)	CS-EDX-CyA-	27	35	—	FSGS (29) FSGS (34)
18a	p.[R229Q]+ [R291W]	North Africa	0.6	Yes (0.6)	CS-Lev-	14	10	—	Not done
18b	p.[R229Q]+ [R291W]	North Africa	0.8	Yes (0.8)	CS-Chl	16	15	—	MCD (1) FSGS (2)
19a	p.[R229Q]+ [A288T]	Europe	15	Yes (24)	CS-CyA-EDX-	7.5	—	50 (31)	MCD (16) MCD (24)
19b	p.[R229Q]+ [A288T]	Europe	15	No	No treatment	—	—	>80 (36)	Not done
20a	p.[R229Q]+ [A288T]	Europe	12	Yes (19)	CS-CyA ± (w/o ACEI)	19	—	>80 (23)	FSGS (19)
21a	p.[R229Q]+ E310A]	Europe	18	Yes (33)	No treatment	—	34	Unknown	FSGS 20) FSGS (27)
22a	p.[R229Q]+ [E310K]	Europe	Childhood	No	No treatment	Unknown	—	60 (35)	FSGS (34)
22b	p.[R229Q]+ [E310K]	Europe	Childhood	Yes (34)	No treatment	Unknown	—	40 (34)	FSGS (34)
23a	p.[R229Q]+ [R138Q]	Europe	8	Yes (39)	CS-MMF-FK-	98	40	—	MCD (39) FSGS (39)
24a	p.[R229Q]+ [A297V]	North Africa	34	Yes (35)	No treatment	11.5	—	34 (36)	UG (35)

Table 4 | Continued

ID	Mutation	Ethnic origin	Disease onset (years)	NS onset (years)	Therapy/ effect	eGFR decline (ml/min/year)	Age at dialysis (years)	eGFR last visit (years)	Histology age (years)
24b	p.[R229Q]+ [A297V]	North Africa	14	No	CS-	Unknown	—	74 (22)	UG (22)
25a	p.[R229Q]+ [R322G]	South America	34	Yes (34)	CS-	Unknown	38	—	FSGS (34)
26a	p.[R229Q]+ [E310K]	Europe	Unknown	Yes (0.2)	CS-CyA-	—	—	Normal (5)	FSGS (0.5)
27a	p.[R229Q]+ [Q215X]	Europe	0.8	Yes (0.8)	CS-EDX-	Unknown	43.5	—	MCD (?) FSGS (38)

ACEI, angiotensin-converting enzyme inhibitors; Chl, chlorambucil; CS, steroids; CyA, cyclosporin A; EDX, cyclophosphamide; eGFR, estimated glomerular filtration rate; FK, FK506; IGL, ischemic glomerular lesions; Lev, levamisole; MMF, mycophenolate mofetil; NS, nephrotic syndrome; UG, unclassified glomerulopathy.
Therapy effect categories: (—) no response; (±) partial reduction of proteinuria. Histology (years): age at which kidney biopsy was performed.

deleterious (PolyPhen), and these changes were not found in 106 controls. Other mutations found in association with p.R229Q within this group have been described elsewhere.[10,24]

Patients carrying p.R229Q and one *NPHS2* mutation developed NS significantly later than those carrying two pathogenic mutations (median 19.0 vs 1.1 years; $P<0.01$). No significant differences were found in the age at onset of NS or ESRD when comparing patients carrying the p.A284V mutation vs other mutations. Three cases with subnephrotic proteinuria were included in this group; all were familial cases with an affected sibling who had developed NS earlier.

In the subset of patients with adult-onset NS ($n=119$), 18 cases from 15 families carried mutations, and all of them were compound heterozygous for the p.R229Q variant and one pathogenic mutation. Overall, the frequency of *NPHS2* mutations in adults was 11% (9/81) in sporadic cases and 25% (6/36) in familial forms. The proportion of patients with adult-onset SRNS carrying *NPHS2* mutations was higher among cases from South America (sporadic cases 4/23; familial forms 3/7) and Europe (sporadic cases 5/43; familial forms 2/11). Adult patients carrying *NPHS2* mutations had an earlier onset of NS compared with those without *NPHS2* mutations (24.9 ± 6 vs 30.0 ± 11 years; $P=0.008$); however, no difference was observed in the age at onset of ESRD (31.9 ± 4 vs 35.1 ± 12 years).

Overall, 27 out of 36 patients received immunosuppressive therapy. The remainder were treated with antiproteinuric drugs; however, steroids were not prescribed either because they had familial SRNS or because they were diagnosed at ESRD. A single patient (20a) experienced a sustained reduction of proteinuria to subnephrotic levels after few weeks of treatment with steroids and cyclosporin A. Angiotensin-converting enzyme inhibitors showed inconsistent effects in the majority of this group; however, in two patients (3a and 3b), these agents dramatically decreased proteinuria from 5 to 0.26 g/day and from 11.5 to 0.27 g/day, respectively. The decline in estimated glomerular filtration rate (eGFR) was variable between patients (from 1 to 98 ml/min per 1.73 m^2 per year): an accelerated rate was observed in at least five cases; the most dramatic decrease was observed in patient 23a who had an eGFR falling from 116 to 18 ml/min within 1 year. Intrafamilial variation in disease progression was also observed: siblings 15a and 15b had normal eGFR at diagnosis; however, one of them slowly progressed toward ESRD, whereas the other conserved a normal GFR after 8 years of follow-up. ESRD was reached in 17 patients at a median age of 27.9 years (range 9.3–43.5), significantly later than the group with two pathogenic mutations (median 8 years; $P<0.001$). Out of nine patients who were transplanted, one developed nephrotic-range proteinuria shortly after transplantation (23a); however, transplant biopsy showed evidence of membranous nephropathy-like lesions, supporting the hypothesis of a *de novo* glomerulopathy as the most probable cause of recurrence of proteinuria.

Disease in p.R229Q homozygotes is variable and shows incomplete penetrance

Eight patients from five families were included in this group (Table 5). Segregation analysis was consistent with an autosomal-recessive disease, but with incomplete penetrance (Figure 2a). The index case of family 29 (II.2) developed SRNS and reached ESRD at 6 and 11 years of age, respectively. Her sibling (II.3), also homozygous for the p.R229Q variant, was asymptomatic at 9 years of age. Overall, these patients presented with nephrotic-range proteinuria between birth and 13 years of age. Kidney biopsy revealed minimal change disease in five of seven patients in which this procedure was performed. A follow-up biopsy was performed in three patients and revealed FSGS in two of them. A sustained reduction of proteinuria to subnephrotic levels was obtained in three cases with steroids and cyclosporin A (28a, 29a, and 30a).

Variable degrees of disease severity were observed. In family 31 (Figure 2b), one patient (31a: II.1) progressed to ESRD at 14 years of age, whereas her haploidentical sibling (31b: II.4) had normal GFR at 26 years of age. In this family, one additional sibling (II.3) presented with mild proteinuria and was found to be heterozygous for p.R229Q. In view of the clinical observations in this family and to search for mutations in other genes that might be the cause of SRNS, we sequenced the entire coding region of *NPHS1*, *PLCE1*,

Table 5 | Ethnic origin, phenotype, and kidney histology in homozygous p.R229Q patients

ID	Ethnic origin	Disease onset (years)	NS onset (years)	Therapy/ effect	eGFR decline (ml/min per year)	Age at dialysis (years)	eGFR last visit (years)	Histology age (years)
28a	Europe	13	Yes (13)	CS-CyA ± MMF-	8.6	—	45 (17)	MCD (13) FSGS (14)
29a	North Africa	6	Yes (6)	CS-CyA ±	15	11	—	MCD (6)
30a	Europe	2.5	Yes (2.5)	CS-CyA ± Chl-	Unknown	15	—	MCD (4) FSGS (8)
31a	Europe	1	Yes (1)	CS-	50	14	—	MCD (2)
31b	Europe	2	Yes (2)	CS-	—	—	>80 (26)	MCD (2) MCD (10)
32a	Middle-East	6.6	Yes (6.8)	CS-CyA-EDX-	Unknown	10.8	—	FSGS (7.6)
32b	Middle-East	Unknown	Yes (unknown)	No treatment	Unknown	14	—	Not done
32c	Middle-East	6	Yes (7)	CyA-	Unknown	—	>60 (15.5)	FSGS (6.8)

Chl, chlorambucil; CS, steroids; CyA, cyclosporin A; eGFR, estimated glomerular filtration rate; MMF, mycophenolate mofetil; NS, nephrotic syndrome.
Therapy effect categories: (−) no response; (±) partial remission. Histology (years): age at which kidney biopsy was performed.

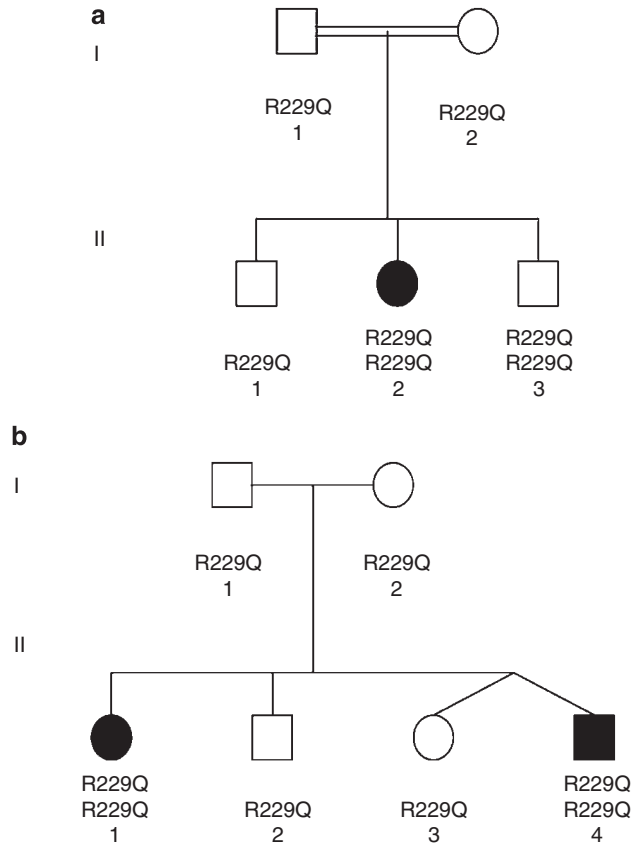

Figure 2 | The p.R229Q homozygotes may present with incomplete penetrance and variable disease severity. (**a**) Incomplete penetrance in cases carrying p.R229Q in the homozygous state is depicted in family 29. Individual II.2, homozygous for p.R229Q, presented with NS at 6 years of age and reached ESRD 5 years after diagnosis. Individual II.3 had no renal dysfunction at 9 years of age. (**b**) Intrafamilial variability and evidence suggesting the involvement of an additional gene in the phenotype of p.R229Q homozygotes is illustrated in family 31. Individual II.1 presented with NS at 7 months of age and reached ESRD at 14 years of age. Individual II.4 was diagnosed with NS at 10 years of age and 16 years later remained with a normal GFR. As shown, both are homozygous for the p.R229Q variant. Unexpectedly, individual II.3, who carries a single p.R229Q allele, had sustained subnephrotic proteinuria with normal GFR. No mutations in *NPHS1*, *NPHS3*, *TRPC6*, or *CD2AP* were found in this family. ESRD, end-stage renal disease; GFR, glomerular filtration rate; NS, nephrotic syndrome.

TRPC6, and *CD2AP* in one of the affected cases, but we did not find additional mutations. In addition, linkage to the *NPHS1* and *NPHS3* loci was excluded in families 29 and 32.

The phenotype of SRNS patients with heterozygous *NPHS2* variants does not differ from that of those without *NPHS2* mutations

The age at presentation of patients carrying a single heterozygous *NPHS2* mutation did not differ significantly from that of those without pathogenic mutations (median 6.5 vs 7.9 years, respectively). Similarly, when comparing heterozygotes for p.R229Q and one wild-type allele with those without mutations, neither the age at onset of NS, nor the age at ESRD differed significantly between groups, although there was a trend toward later disease onset among those carrying a single p.R229Q allele (Table 1). Further statistical analyses were limited by the small sample size.

DISCUSSION

In this study, we examined the role of the podocin p.R229Q variant in a large and ethnically diverse cohort of patients with SRNS. To precisely define our study group, we excluded cases with SRNS due to a potential underlying immune disorder; thereby selecting those patients in whom a presumed podocyte structural abnormality was the most likely cause of the disease. We found epidemiological and clinical evidence to affirm that the p.R229Q variant is implicated in the pathogenesis of SRNS. The frequency of the p.R229Q allele was significantly higher in SRNS patients than in controls, particularly in European or South American populations. More interestingly, the fact that patients carrying one *NPHS2* pathogenic mutation were more likely to be compound heterozygous for p.R229Q than to carry a wild-type allele supports the plausible pathogenic role of this association in the development of SRNS. Indeed, single heterozygous for p.R229Q or a pathogenic *NPHS2* mutation did not exhibit a phenotype different than that in those patients carrying two wild-type alleles. This observation is concordant with the abundant clinical evidence indicating that SRNS due to podocin mutations follows an autosomal-recessive pattern of inheritance.[10,20,23,24,28–30] Moreover, in *Nphs2* mouse models, we have shown that animals develop

NS only if they are homozygous for a null allele [31,32] or for a point missense mutant allele,[33] but that heterozygotes have no renal phenotype.

The p.R229Q variant was found in the compound heterozygous state in 36 cases in whom the onset of NS and ESRD was significantly later than in those with two pathogenic mutations. A mutation (p.A284V) already described in late-onset FSGS predominated in the group of patients heterozygous for p.R229Q (mostly from Chile and Argentina).[21,24,29] *In silico* analysis of this substitution suggests that it is likely pathogenic. Another line of evidence pointing to the importance of alanine at position 284 is its conservation within podocin orthologs. Finally, we did not find it in 308 European and 70 South American controls. The high frequency of p.A284V associated with the p.R229Q variant in a South American population and with adult-onset SRNS should lead to genotyping for these changes in larger cohorts to confirm our findings.

Confirmation of the role of *NPHS2* mutations in adult-onset disease has been limited essentially because only few cases have been identified.[24,34–37] We show here that patients presenting with SRNS after 18 years of age can have *NPHS2* mutations, as in our cohort, 11% of the sporadic cases and 25% of the familial forms carried one disease-causing mutation in the compound heterozygous state with the p.R229Q variant. Our conclusions support the results initially obtained by Tsukaguchi *et al.*[24] who reported that the frequency of *NPHS2* mutations in late-onset familial SRNS reached 23.3%. Results from three large cohorts published subsequently are discordant with our findings. Caridi *et al.*[28] did not find *NPHS2* mutations among 64 adults with SRNS. More recently, He *et al.*[34] screened 87 cases with adult-onset idiopathic FSGS. In the steroid-resistant subgroup, the mean age of onset was 38 years. Mutations were found in only one patient (p.R229Q + p.Q285fsX302) who presented with NS at 38 years of age and unexpectedly had a complete sustained remission after steroids (2 years follow-up). Intriguingly, this is the first patient with *NPHS2* mutations known to achieve a full remission after steroids. Earlier screening in patients with steroid-dependent, frequent-relapsing, late steroid-resistant, and steroid-sensitive NS has failed to identify individuals carrying mutations.[38,39] In addition, we have not detected *NPHS2* mutations in 120 cases with either response to immunosuppressive therapy or post-transplant recurrence (unpublished data). Finally, McKenzie *et al.*[37] examined 265 patients with late-onset FSGS including those with underlying immune disorders; however, no *NPHS2* mutations were found. To interpret these results in view of our own, it is crucial to consider that our cohort is highly selective, as we excluded cases with a presumed immune etiology. In the McKenzie cohort, over 60% of the patients were African-Americans, who carry the p.R229Q allele in a very low frequency.[25] Moreover, SRNS patients carrying *NPHS2* mutations have not been found in this ethnic group.[25,37,40] Certainly, these issues should be considered when making decisions regarding mutational screening.

Our findings raise important issues regarding genetic counseling in families with *NPHS2* mutations, as compound heterozygotes with the p.R229Q variant on one allele and a pathogenic *NPHS2* mutation on the other develop progressive glomerular disease.[20,24–26] Information on the frequency and potential role of p.R229Q should be given to affected families. Screening for p.R229Q should be proposed to spouses of either patients bearing p.R229Q associated with a pathogenic *NPHS2* mutation or spouses of known heterozygous carriers of *NPHS2* mutations.

The role of p.R229Q homozygosity in SRNS is less well defined. In Europeans, we found a slight increase in the proportion of homozygous SRNS patients than controls (3/214 vs 0/308 unrelated individuals). An analysis of four previous reports performing *NPHS2* mutational screening in Caucasians (mostly from Europe and North America) comprising a total of 819 unrelated SRNS patients and 4695 controls revealed that the proportion of homozygous individuals was remarkably higher among affected cases than among controls (7/819 vs 4/4695; $P = 0.0003$).[23,27,37,41] This observation suggests that p.R229Q homozygosity may increase the risk for SRNS, although the magnitude of this effect remains unknown. The minor allele frequency of p.R229Q in our cohort of European controls was 0.026, meaning that one would expect to find one homozygote per 1000 individuals. Therefore, the expected frequency of homozygotes is much higher than the frequency of SRNS in the Caucasian population. More likely, p.R229Q in the homozygous state may act as a disease modifier, predisposing individuals to develop NS following an initial renal insult. Mutations in other genes important for the glomerular filtration barrier are probably responsible for the phenotype in these patients. The finding of microalbuminuria in one heterozygous individual, the partial response to immunosuppressive therapy observed in some homozygous cases, and the finding of an asymptomatic p.R229Q homozygous sibling strengthen the hypothesis of p.R229Q homozygosity as a disease modifier in these families. In conclusion, *NPHS2* mutations were not uncommon in our cohort of patients with juvenile and adult forms of SRNS. Most of the cases were Europeans or South Americans of Spanish descent and carried the p.R229Q variant in association with a pathogenic mutation. First-step screening for p.R229Q should be proposed in Caucasian adolescents and young adults with SRNS in whom there is no evidence of response to immunosuppressive therapy or relapse after transplantation, especially if they are from these particular ethnic populations. Only in those cases carrying p.R229Q would further analysis to identify a mutation of *NPHS2* on the second allele be warranted. This screening has three clinical goals: (1) to avoid unnecessary immunosuppressive treatment; (2) to promote living related donor kidney transplantation, because the risk of recurrence of FSGS in the graft is much lower in *NPHS2* disease than in primary FSGS, and (3) to provide accurate genetic counseling to the patients and their families.[20,21] The significance of p.R229Q homozygosity remains to be

clarified, and although it does not appear pathogenic on its own, it may serve as a disease modifier in NS.

MATERIALS AND METHODS

Patients

From a worldwide cohort of 747 patients with autosomal-recessive and sporadic SRNS referred for *NPHS2* genotyping, we selected 546 patients belonging to 455 unrelated families for genetic analysis. We excluded cases achieving remission after immunosuppressive therapy ($n=50$), as well as those with secondary steroid resistance ($n=27$) or with post-transplant recurrence ($n=70$), as we considered that an underlying immune disorder was the most likely cause of the disease in these cases. Individuals with extrarenal manifestations ($n=15$), isolated subnephrotic proteinuria ($n=20$), or insufficient clinical information ($n=19$) were also excluded. Patients originating from a consanguineous marriage and/or those with an additional affected sibling were considered as familial cases. To calculate frequencies of mutations we used the number of families; when evaluating phenotypes, we considered individual data. We reviewed medical information to obtain the age of first urinary abnormality, onset of NS, course of glomerular disease, and renal histopathological findings. Recurrence after kidney transplantation was assessed as well. eGFR was calculated by using the abbreviated modified of diet in renal disease (MDRD) formula in adults and Schwartz formula in children.[42,43] Diagnosis of NS and the assessment of treatment response were performed at each referring center by nephrologists in accordance with criteria published earlier.[2,44] Out of the 546 patients in our cohort, 303 were studied earlier with respect to genetic heterogeneity and post-transplant recurrence in cases with *NPHS2* mutations.[20]

Genotyping and mutation analysis

Genomic DNA was isolated from peripheral blood by standard methods after obtaining informed consent from affected individuals or their parents. The complete coding sequence and exon–intron boundaries of the *NPHS2* gene were amplified by PCR;[10] subsequently, both strands were sequenced using a BigDye terminator cycle sequencing kit and analyzed with an ABI Prism 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA). Segregation of mutations with disease was assessed by direct sequencing from all available family members. Linkage to the *NPHS2* locus was assessed using microsatellite markers *D1S3760*, *D1S215*, *D1S3759*, and *D1S2751*. In individuals homozygous for p.R229Q, sequencing of *NPHS1*, *TRPC6*, *CD2AP*, and *PLCE1* was performed whenever linkage was confirmed. Unpublished missense mutations were screened in at least 100 unrelated controls either by direct sequencing or by single-base extension using SNaPshot Multiplex kit (Applied Biosystems). *In silico* analyses of missense mutations found in compound heterozygous state with p.R229Q were performed using the PolyPhen software.[45,46]

Renal histology

Biopsy specimens for light microscopy and immunofluorescence were analyzed following standard techniques. Kidney biopsy report was required for all the cases derived from a referring center.

Statistical analysis

All values are expressed as means ± s.d. or median and range. Comparisons between two continuous variables were made using the Mann–Whitney *U*-test. For categorical variables, testing for difference in proportions was performed using the χ^2 or the Fisher's exact test when indicated. All tests were two sided. *P*-values <0.01 were considered significant. Statistical analyses were performed using Minitab 13.0 software (Minitab, State College, PA, USA).

DISCLOSURE

All the authors declared no competing interests.

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