Pediatr Nephrol (2004) 19:1340–1348
DOI 10.1007/s00467-004-1629-3

ORIGINAL ARTICLE

Michael Schultheiss · Rainer G. Ruf · Bettina E. Mucha · Roger Wiggins · Arno Fuchshuber · Anne Lichtenberger · Friedhelm Hildebrandt

No evidence for genotype/phenotype correlation in *NPHS1* and *NPHS2* mutations

Received: 23 February 2004 / Revised: 19 July 2004 / Accepted: 20 July 2004 / Published online: 24 August 2004

Members of the Study Group of the Arbeitsgemeinschaft für Pädiatrische Nephrologie (APN) participating in this study: J. Thaarup (Aalborg, Denmark); P. Henning (Adelaide, Australia); I. Attrach (Aleppo, Syria); A. Bakkaloglu (Ankara, Turkey); C. Rudin (Basel, Switzerland); R. Bogdanovic (Belgrade, Yugoslavia); S. Briese, J. Gellermann, T. Lennert, U. Querfeld, Sacherer, M. Schürmann, and M. Zimmering (Berlin, Germany); C. Roth, C. Schröter, and B. Utsch (Bonn, Germany); Matthes (Bremen, Germany); A. Heilmann and G. Kalvoda (Dresden, Germany); F. Wegner (Düren, Germany); V. Schumacher (Düsseldorf, Germany); Bär, B. Bosch, M. Kamm, S.M. Karle, K. Nüsken, C. Plank, W. Rascher, and B. Zimmermann (Erlangen, Germany); K. E. Bonzel, M. Bald, P. Hoyer, and U. Vester (Essen, Germany); U. Neyer (Feldkirch, Austria); Rippel (Frankfurt, Germany); M. Brandis, A. Fuchshuber, K. Häffner, A. Kirchhoff, and M. Pohl (Freiburg, Germany); J. Steiss (Giessen, Germany); J.P. Haas (Greifswald, Germany); L. Patzer (Halle, Germany); M. Kemper, H. Altrogge, D.E. Müller-Wiefel, U. Peters, and K. Timmermann (Hamburg, Germany); J.H.H. Ehrich, H. Haller, and C. Strehlau (Hannover, Germany); M. Daschner, S. Hessing, Janssen, D. Kiepe, S. Köpf, O. Mehls, and B. Tönshoff (Heidelberg, Germany); F. Prüfer and L.B. Zimmerhackl (Innsbruck, Austria); U. John, J. Misselwitz, G. Rönnefarth, and J. Seidel (Jena, Germany); D. Blowey and J. Scheinman (Kansas City, Mo., USA); B. Beck, K. Frankenbusch, B. Hoppe, C. Licht, D. Michalk, T. Ronda, and L. Stapenhorst (Cologne, Germany); D. Drozdz and A. Pogan (Krakau, Poland); Froster, E. Vogel and S. Wygoda (Leipzig, Germany); R. Hettenger (Los Angeles, Calif., USA); H. Schriewer and H.-P. Weber (Lüdenscheid, Germany); R. Beetz (Mainz, Germany); M. Konrad (Marburg, Germany); H. Fehrenbach (Memmingen, Germany); M. Griebel and B. Klare (München, Germany); M. Bulla, S. Fründ, E. Kuwertz-Bröking, A. Schulze-Everding and Yelbuz (Münster, Germany); L. Monnens (Nijmegen, The Netherlands); J. Janda and T. Seemann (Prag, Czech Republic); G. Adomssent, G. Krüger, Lorenzen, J. Muscheites, H.-J. Stolpe and M. Wigger (Rostock, Germany); W. Sperl (Salzburg, Austria); R. Egger (Schaffhausen, Switzerland); V. Tasic (Skopje, Macedonia); M. Bald and H.-E. Leichter (Stuttgart); O. Amon (Tübingen, Germany); T. Arbeiter, C. Aufricht and K. Müller (Vienna, Austria); D. Bockenhauer and N. Siegel (New Haven, Conn., USA); and T. Neuhaus and A. Staub (Zürich, Switzerland)

M. Schultheiss · R. G. Ruf · B. E. Mucha · F. Hildebrandt
Department of Pediatrics,
University of Michigan,
Ann Arbor, Michigan, USA

F. Hildebrandt
Department of Genetics,
University of Michigan,
Ann Arbor, Michigan, USA

R. G. Ruf
Department of Cardiology,
Franz-Volhard-Klinik,
Charité, Berlin-Buch, Germany

R. Wiggins
Department of Internal Medicine,
University of Michigan,
Ann Arbor, Michigan, USA

A. Fuchshuber · A. Lichtenberger
Department of Pediatrics,
University of Freiburg,
Freiburg, Germany

F. Hildebrandt (✉)
University of Michigan Health System,
8220C MSRB III, 1150 West Medical Center Drive,
Ann Arbor, MI 48109–0646, USA
e-mail: fhilde@umich.edu
Tel.: +1-734-6157285
Fax: +1-734-6151386

Abstract Primary steroid-resistant nephrotic syndrome (SRNS) is characterized by childhood onset of proteinuria and progression to end-stage renal disease. In 26% of cases it is caused by recessive mutations in *NPHS2* (podocin). Congenital nephrotic syndrome (CNS) is caused by mutations in *NPHS1* (nephrin) or *NPHS2*. In three families mutations in *NPHS1* and *NPHS2* had been reported to occur together, and these tri-allelic mutations were implicated in genotype/phenotype correlations. To further test the hypothesis of tri-allelism, we examined a group of 62 unrelated patients for *NPHS1* mutations, who were previously shown to have *NPHS2* mutations; 15 of 62 patients had CNS. In addition, 12 CNS patients without *NPHS2* mutation were examined for *NPHS1* mutations. Mutational analysis yielded three different groups. (1) In 48 patients with two recessive *NPHS2* mutations

(11 with CNS), no *NPHS1* mutation was detected, except for 1 patient, who had one *NPHS1* mutation only. This patient was indistinguishable clinically and did not have CNS. (2) In 14 patients with one *NPHS2* mutation only (4 with CNS), we detected two additional recessive *NPHS1* mutations in the 4 patients with CNS. They all carried the R229Q variant of *NPHS2*. The CNS phenotype may be sufficiently explained by the presence of two *NPHS1* mutations. (3) In 12 patients without *NPHS2* mutation (all with CNS), we detected two recessive *NPHS1* mutations in 11 patients, explaining their CNS phenotype. We report ten novel mutations in the nephrin gene. Our data do not suggest any genotype/phenotype correlation in the 5 patients with mutations in both the *NPHS1* and the *NPHS2* genes.

Keywords Steroid-resistant nephrotic syndrome · Congenital nephrotic syndrome · *NPHS1* · *NPHS2* · Mutational analysis

Introduction

Nephrin and podocin, the protein products of the *NPHS1* and *NPHS2* genes, directly interact via their C-terminal domains in a protein-protein interaction pathway that is responsible for maintaining glomerular podocyte foot process integrity and function [1, 2]. The presence of two recessive mutations in the *NPHS1* and *NPHS2* genes that impair function of these proteins results in altered podocyte structure and function. The glomerular filtration barrier to protein is then defective [3]. The mechanistic consequence (phenotype) is that protein leaks from blood into the urinary space to be lost from the body in the urine, thereby causing the nephrotic syndrome (NS). There has been increasing realization that heterozygous mutations in more than one gene coding for proteins involved in important protein-protein interaction pathways may together impact the phenotype, and therefore affect clinical disease, including age of onset, severity, and outcome [4]. These associated heterozygous mutations might contribute to a genetic modifier effect well recognized to be important in determining the ultimate phenotype. This additional level of genetic complexity is superimposed on already complex factors such as the functional consequence of a mutation.

The range of phenotypes in childhood NS has traditionally been described in relation to age at onset. Additional pathological features derived from renal biopsy are superimposed on this clinical classification. Steroid-resistant nephrotic syndrome (SRNS) has been described as childhood onset of proteinuria, rapid progression to end-stage renal disease (ESRD), resistance to standard steroid therapy, and absence of recurrence after renal transplantation [5]. In SRNS about 75% of patients exhibit renal histology of focal segmental glomerulosclerosis (FSGS) and 20% show minimal change nephrotic syndrome (MCNS). Following the identification of the *NPHS2* gene encoding podocin (OMIM 604766) [6], different groups have demonstrated that mutations in the *NPHS2* gene represent a frequent rather than a rare cause of SRNS, since they occur in approximately 26% of unrelated patients with SRNS [7, 8]. It has been proposed, based on a very small number of patients [6], that SRNS patients with two recessive mutations in *NPHS2* do not respond to standard steroid treatment. We recently confirmed this hypothesis in 190 patients with SRNS and 124 patients with steroid-sensitive nephrotic syndrome (SSNS). We showed that SRNS patients with two recessive mutations in *NPHS2* have a reduced risk for recurrence of FSGS in a renal transplant compared with children without mutations (8% compared with 35%) [8].

Congenital nephrotic syndrome (CNS) is a clinical syndrome where proteinuria and edema are present before birth and are associated with a large placenta, prematurity, and marked edema at birth [9]. Histology shows a wide variability but fibrotic lesions, focal mesangial cell hyperplasia, tubular atrophy, and dilatation of glomerular capillaries are common features [10, 11]. The most frequent cause of CNS is CNS of the Finnish type (CNF), which is an autosomal recessive disorder with a higher incidence in Finland (1:10,000 births) [12] than in other countries [13]. Recessive mutations in *NPHS1*, encoding nephrin (OMIM 602716) [14], are the most frequent cause of CNS (CNF). Of Finnish CNS patients, 94% show one of two mutations: a two-base pair deletion in exon 2 (Fin major) or a nonsense mutation in exon 26 (Fin minor) [14]. An R1160X nonsense mutation was recently found to be a founder mutation in the Maltese population [15]. In other populations no mutation "hot-spots" have been found, and mutations are equally distributed over the *NPHS1* gene [16, 17].

The gene products of the two recessive genes, causing SRNS and CNS, nephrin (encoded by *NPHS1*) and podocin (encoded by *NPHS2*), were identified as being important for the function of the glomerular slit membrane of podocyte foot processes, which constitutes the primary molecular sieve of the glomerulus [18]. Data showing evidence of a functional inter-relationship between nephrin and podocin within the podocyte were reported recently [15] by identifying mutations both in *NPHS1* and *NPHS2* in three families. A di-genic inheritance resulting in a tri-allelic number of mutations was thought to modify the histological phenotype from CNS of the Finnish type (CNF) to congenital FSGS [15].

We therefore examined a cohort of 74 patients with childhood NS for the *NPHS1* and *NPHS2* genes to test for the frequency of mutations in more than one of the two genes and relate the findings to the phenotype, if possible.

We performed mutational analysis in the promoter region and all 29 *NPHS1* exons in 48 patients (11/48 presented as CNS) in whom we had detected two recessive *NPHS2* mutations and in 14 patients (4/14 presented as CNS) in whom we had detected one heterozygous *NPHS2* mutation only. We also examined 12 CNS patients without *NPHS2* mutation for *NPHS1*. Our primary goal was to determine (1) whether there was any evidence of *NPHS1* mutations in the 63 patients with *NPHS2* mu-

tations, (2) whether there were any genotype/phenotype correlations of combined *NPHS1* and *NPHS2* mutations, (3) whether mutations in *NPHS1* could fully explain the phenotype seen in patients with CNS and no *NPHS2* mutation.

In conclusion, we report ten novel *NPHS1* mutations. We found 5 patients with combined mutations in *NPHS1* and *NPHS2,* but absence of evident genotype/phenotype correlations.

Patients and methods

Patients

Blood samples for mutational analysis, clinical data, and informed consent were obtained from patients or their parents (http://www.renalgenes.org). Genomic DNA was isolated directly from blood samples by standard methods [19] using the QIAamp blood kit (Qiagen, Valencia, Calif., USA). Ethic committee approval was obtained from the ethic commission of the University of Freiburg, Germany and was sought from the University of Michigan, Ann Arbor. The clinical diagnoses of SRNS and/or CNS were established by pediatric nephrologists from different centers of pediatric nephrology according to published criteria [20, 21]. Of the 62 patients with *NPHS2* mutations, 46 had received standard steroid treatment and proved resistant, only 5 did not receive steroids. Of these 5 patients, 3 had congenital onset, for the other 2 the reason for not receiving steroids is unknown. For 11 patients no data on the treatment modalities were available, but no patient was reported to be steroid sensitive. Of the 12 CNS patients without *NPHS2* mutations, 3 had received standard steroid treatment and proved resistant, in 8 patients no standard steroid treatment was performed, and for 1 patient no data on the treatment modalities were available. Three groups of patients were screened for *NPHS1* mutations: patients with two recessive *NPHS2* mutations, patients with one heterozygous *NPHS2* mutation only, and CNS patients with no *NPHS2* mutation. In the third group, 2 patients (F475 II-1 and F1328 II-1) also had one affected sibling. Since these siblings showed a similar phenotype we have not included them in the number calculations for clinical data. Patients of groups I, II, and III with mutations in the *NPHS1* gene were divided into subgroup A (group I patients), subgroup B (group II patients), and subgroup C (group III patients) in Table 1. For clinical evaluation we used a standard questionnaire (http://www.renalgenes.org) as previously described [7]. Characteristic features defining the clinical diagnosis were: age of onset, initial symptoms, histology of the kidney biopsy, progression to ESRD, and renal transplantation (Table 1). None of the patients showed significant extrarenal manifestations of other organ systems. CNS was defined as the presentation of NS within the first 2 months of life, because one-quarter of the CNF children presented with edema or abdominal distention at birth, another quarter presented during the 1st week of life, and all untreated patients developed full-blown NS before the age of 3 months [21]. CNF in kidney biopsy usually presents as proximal tubular dilatation and diffuse glomerular mesangial hypercellularity at an early stage. Later biopsies show mesangial hypercellularity and degenerative changes such as fibrotic thickening of the Bowman's capsule and interstitial fibrosis [15, 21, 22]. In the patients with two recessive *NPHS1* mutations (Table 1, subgroup B and subgroup C), consanguinity was known for 8 of 15 (53%). The ethnic background of the patients was central European, Turkish, and Arabic.

Mutational analysis

Mutational analysis for *NPHS1* was performed by direct sequencing of the promoter region and of all 29 exons of the *NPHS1* gene for one strand. For all 18 amplicons (compromising the promoter region and all 29 exons), the forward primers were used for sequencing as described previously [23, 24]. All mutations were confirmed by sequencing the complementary strand. Known single nucleotide polymorphisms within the primer sequences were avoided (http://genome.ucsc.edu/), because single nucleotide variants within the primer sequence can suppress amplification of one of the two alleles of the amplified product. To rule out polymorphisms, at least 160 chromosomes of 80 healthy control individuals (age and ethnic background similar to the patients screened for mutations in *NPHS1)* were checked for novel mutations by restriction enzyme digestion and denaturing high-performance liquid chromatography. For sequence evaluation the program SEQUENCHER version 4.1.4 (GeneCode, Ann Arbor, Mich., USA) was used. All patients had undergone previous mutational analysis of podocin [8] and exons 6, 7, 8, and 9 of the *WT1* gene (data not shown, Ruf et al., in press). None of the patients in this study showed mutations in exons 6, 7, 8, and 9 of the *WT1* gene.

Results

Clinical data and summary of results

The study included 75 unrelated patients with NS. Clinical data are shown in Tables 1 and 2. Renal biopsy results in patient group I were FSGS 28 of 48 (58%), MCNS 9 of 48 (19%), mesangioproliferative glomerulonephritis 2 of 48 (4%), IgM nephropathy 1 of 48 (2%), Alport nephropathy 1 of 48 (2%), CNF 1 of 48 (2%), and no data or no biopsy performed 6 of 48 (13%) (Table 2). Renal biopsy results in patient group II were FSGS 5 of 14 (36%), MCNS 5 of 14 (36%), diffuse mesangial sclerosis (DMS) 1 of 14 (7%), and no data or no biopsy performed 3 of 14 (21%) (Table 2). Renal biopsy results in patient group III were FSGS 2 of 12 (17%), MCNS 2 of 12 (17%), DMS 1 of 12 (8%), CNF 2 of 12 (17%), and no data or no biopsy performed 5 of 12 (41%) (Table 2). The median age at onset was 2.4 years in patient group I (median 3.3 years after subtracting the 11 CNS patients), 1.3 years in patient group II (median 4.0 years after subtracting the 4 CNS patients), and 0.0 years in patient group III. CNS was diagnosed in 11 of 48 (23%) patients of group I and 4 of 14 (29%) patients of group II; 23 of 48 (48%) patients of group I progressed to ESRD with a median age of 9.3 years; 3 of 14 (21%) patients of group II progressed to ESRD with a median age of 13.0 years; 5 of 12 (42%) patients of group III progressed to ESRD with a median age of 3.4 years (Table 2). There were 48 patients with two recessive *NPHS2* mutations (Table 2, group I), but only 1 of them showed an additional heterozygous *NPHS1* mutation (Table 1, subgroup A). There were 14 patients with one heterozygous *NPHS2* mutation only (Table 2, group II); 4 of these patients had two additional recessive *NPHS1* mutations (Table 1, subgroup B). Twelve CNS patients showed no *NPHS2* mutation (Table 2, group III), but 11 of them had two recessive *NPHS1* mutations (Table 1, subgroup C).

Table 1 Clinical data of patients with mutations in *NPHS1* (*CNS* congenital nephrotic syndrome, *FSGS* focal segmental glomerulosclerosis, *MCNS* minimal change nephrotic syndrome, *ESRD* end-stage renal disease, *KTx* kidney transplantation, *ND* not done, *?* no data available, *DMS* diffuse mesangial sclerosis, *AS* acute symptoms, *CNF* histological picture of CNS of the Finnish type)

Sub-group[a]	Patient	Gender	Mutation in *NPHS1* (effect on translation product)	Mutation in *NPHS2* (effect on translation product)	Age of onset (years)	Initial symptoms	Parental consan-guinity	Biopsy	ESRD (evaluated years after onset)	KTx (evaluated years after onset)
A	F1268	F	G2971C (V991L)[b],h	insT460–467 (V165X), H G709C (E237Q), H	1.6	AS	?	ND	No (1.3)	No
B	F435	M	delTCAinsCC2617 (L904X), H C2552T (A851V), H	G686A (R229Q), h	0.0	AS	No	MCNS (CNF?)	?	?
B	F451	M	C468G (Y162X), h IVS27+1G>T, h	G686A (R229Q), h	0.0	AS	No	ND	?	?
B	F1296	F	delTCAinsCC2617 (L904X), H C2552T (A851V), H	G686A (R229Q), h	0.0	AS	Yes	ND	No (?)	No
B	A140	M	C65T (A22V) b, H	G686A (R229Q), h	0.0	?	?	DMS	Yes (1.3)	Yes (3.8)
C	F212	M	C1135T (R379W)[b], H	No	0.0	?	Yes	?	?	?
C	F228	M	T1738G (W580G)[b], h C2479T (R827X)[b], h	No	0.0	AS	No	DMS	Yes (3.4)	Yes (4.2)
C	F294	M	IVS20+2T>A[b], H	No	0.0	AS	Yes	ND	Yes (3.0)	Yes (4.0)
C	F439	M	delC1814 (V621X)[b], H	No	0.0	AS	Yes	ND	?	?
C	F475 II-1/II-2	F/F	delTCAinsCC2617 (L904X), H C2552T (A851V), H	No	0.0/0.0	AS/AS	Yes	MCNS/yes ?	Yes (1.8)/yes (3.8)	Yes (2.8)/?
C	F571	F	G1379A (R460Q), H	No	0.1	AS	No	MCNS	No (2.0)	No
C	F626	F	delACC514 (delT172), H	No	0.0	AS	Yes	ND	No (0.2)	No
C	F806	F	C3478T (R1160X), H	No	0.1	AS	No	CNF	Yes (7.7)	Yes (9.0)
C	F1017	M	T1258G (F420V)[b], h delCT121 (A90X), h	No	0.0	AS	No	CNF	No (9.0)	No
C	F1093	F	G2014C (A672P)[b], H	No	0.1	AS	Yes	Yes ?	?	?
C	F1328 II-1/II-3	M/F	C385T (L130F)[b], h G1868T (C623F), h	No	0.0/0.0	AS/AS	Yes	FSGS/nor-mal	No (24.0)/no (20.0)	No/no

[a] Subgroup A: patients with one heterozygous *NPHS1* mutation and two recessive *NPHS2* mutations; subgroup B: patients with two recessive *NPHS1* mutations and one heterozygous *NPHS2* mutation; subgroup C: patients with CNS, two recessive *NPHS1* mutations and no *NPHS2* mutation

[b] Novel mutation

Table 2 Clinical and mutational analysis in 48 patients with steroid-resistant nephrotic syndrome (SRNS) with two recessive *NPHS2* mutations, 14 SRNS patients with only one heterozygous *NPHS2* mutation, and 12 CNS patients without *NPHS2* mutation (*ND* not done, *NA* not applicable)

Group	Number of patients	Median age of onset	Biopsy FSGS/MCNS/other/ND	ESRD	Median age at ESRD	KTx	Median age at KTx
		(years)	(%)	(number)	(years)	(number)	(years)
I SRNS patients with 2 recessive *NPHS2* mutations							
2 recessive *NPHS1* mutations	0/48	NA	NA	NA	NA	NA	NA
1 heterozygous *NPHS1* mutation (Table 1, subgroup A)	1/48	1.6	0/0/0/1	0/1	NA	NA	NA
Absence of *NPHS1* mutation	47/48	2.5	*n*=28/9/5/5 (59/19/11/11)	23/47	9.3	18/47	10.7
Total of group I patients	48[a]	2.4[b]	*n*=28/9/5/6 (58/19/10/13)	23/48	9.3	18/48	10.7
II SRNS patients with 1 heterozygous *NPHS2* mutation							
2 recessive *NPHS1* mutations (Table 1, subgroup B)	4[c] /14	0.0	0/1/1/2	1/4	1.3	1/4	3.8
1 heterozygous *NPHS1* mutation	0/14	NA	NA	NA	NA	NA	NA
Absence of *NPHS1* mutation	10/14	4.0	*n*=5/4/0/1 (50/40/0/10)	2/10	13.0	0/10	NA
Total of group II patients	14[d]	1.3[e]	*n*=5/5/1/3 (36/36/7/21)	3/14	13.0	1/14	3.8
III CNS patients with no *NPHS2* mutation							
2 recessive *NPHS1* mutations (Table 1, subgroup C)	11/12	0.0	*n*=1/2/3/5 (9/18/27/46)	4/11	3.2	4/11	4.1
1 heterozygous *NPHS1* mutation	0/12	NA	NA	NA	NA	NA	NA
Absence of *NPHS1* mutation	1/12	0.1	*n*=1/0/0/0	1/1	4.2	0/1	NA
Total of group III patients	12[f]	0.0	*n*=2/2/3/5	5/12	3.4	4/12	4.1

[a] 11/48 patients presented as CNS
[b] Median was 3.3 after subtracting the 11 CNS patients
[c] All 4 patients presented as CNS
[d] 4/14 patients presented as CNS
[e] Median was 4.0 after subtracting the 4 CNS patients
[f] Affected siblings of 2 patients not included

Detection of 10 novel and 9 known *NPHS1* mutations

Since the result of the *NPHS2* mutational analysis has been reported previously [8], we describe here only the results for the *NPHS1* mutational analysis. *NPHS1* mutational analysis showed 19 different mutations in the *NPHS1* gene; 10 were missense mutations, 3 were nonsense mutations, leading to a stop codon, 4 were frameshift mutations, leading to a premature stop codon, and 2 were splice site mutations (Table 1). Ten of the mutations have never been described before and are therefore novel. All novel mutations were absent from 160 chromosomes of healthy control individuals.

The novel mutations include (Table 1): (1) a C65T transition leading to a conservative amino acid exchange A22 V (codon conserved in mice) was found homozygously in A140; (2) a C385T transition leading to a conservative amino acid exchange L130F (codon conserved in mice) was found heterozygously in F1328 II-1 and F1328 II-3; (3) a C1135T transition leading to a non-conservative amino acid exchange R379 W (codon conserved in mice and *Drosophila melanogaster*) was found homozygously in F212; (4) a T1258G transversion leading to a conservative amino acid exchange F420 V (codon conserved in mice) was found heterozygously in F1017; (5) a T1738G transversion leading to a non-conservative amino acid exchange W580G (codon conserved in mice and *Drosophila melanogaster*) was found heterozygously in F228; (6) a G2014C transversion leading to a conservative amino acid exchange A672P (codon conserved in mice and *Drosophila melanogaster*) was found homozygously in F1093; (7) a G2971C transversion leading to a conservative amino acid exchange V991L (codon not conserved in evolution) was found heterozygously in F1268; (8) a C2479T transition leading to a premature stop codon (R827X) was found heterozygously in F228; (9) a deletion of C at position 1814 in the 605th codon inducing a frameshift resulting in a stop codon at V621X was found homozygously in F439; (10) an obligatory splice site mutation IVS20+2T→A involving the 5′ donor splice site of intron 20 was found homozygously in F294.

In 4 patients of three families (F475 II-1, F475 II-2, F435, and F1296) we found homozygously a C2552T transition leading to a conservative amino acid exchange A851 V (codon conserved in mice and *Drosophila melanogaster*). This mutation was not found in any other patient and was absent from 94 healthy control individuals. It only occurred in combination with the homozygous mutation: deletion TCA, insertion CC at position 2617, leading to a stop codon at position 904 (Table 1). Both mutations are localized in exon 19, only 65 base pairs apart from each other. In the healthy mother (F475 I-2) both mutations were found heterozygously. Both mutations were first described by Beltcheva et al. [16], but this publication does not specify if they occur in the same patient. Both mutations were not found in any other patients. The combined appearance of A851V and delTCAinsC2617 is apparently due to linkage disequilibrium. In patient F1017, originally from Germany, we describe the first occurrence of Fin major [delCT121 (A90X)] outside Scandinavia.

Two recessive *NPHS2* mutations in combination with single heterozygous *NPHS1* mutation

In 1 patient with SRNS and two homozygous *NPHS2* mutations (F1268), one heterozygous *NPHS1* mutation was found (Table 1, subgroup A). Since the amino acid exchange V991L is conservative and is not conserved in evolution, and since the patient (F1268) is originally from Oman, this mutation might represent a rare innocuous polymorphism not found in the 80 Caucasian healthy control individuals.

Single heterozygous *NPHS2* mutation in combination with two recessive *NPHS1* mutations

In 4 of 14 patients with one heterozygous *NPHS2* mutation only, two recessive *NPHS1* mutations were found (Table 1, subgroup B). All of these 4 patients had CNS and showed only one heterozygous *NPHS2* mutation with the amino acid exchange R229Q. No mutation in *NPHS1* was found in 10 of 14 patients of this group.

Two recessive *NPHS1* mutations in CNS patients with no *NPHS2* mutation

In 11 of 12 CNS patients with no *NPHS2* mutation, we detected two recessive *NPHS1* mutations (Table 1, subgroup C). These mutations would explain their CNS phenotype.

Single heterozygous mutations detected in *NPHS2*

The single heterozygous *NPHS2* mutations found in 14 patients (Table 2, group II) were described by Ruf et al. [8], but because of the importance of combined *NPHS1* and *NPHS2* mutations, they should be reassessed. In 8 of 14 patients we found the common amino acid exchange R229Q. In 2 of 14 patients we found V290M heterozygously, in 1 of 14 patients we observed R138Q heterozygously and in 1 of 14 patients E310V heterozygously. All 4 occur in compound heterozygous or homozygous mutations in other patients [8] and for R229Q and R138Q clear functional relevance has been demonstrated [25, 26]. There was 1 of 14 patients with the amino acid exchange A242V and 1 of 14 patients Q328R. The significance of these single heterozygous mutations remains unclear.

Discussion

We report ten novel *NPHS1* mutations in children with CNS. Only 5 patients with combined mutations in *NPHS1*

and *NPHS2* were identified. We did not observe any obvious genotype/phenotype correlations regarding age at onset and histological presentation.

Koziell et al. [15] first reported mutations both in *NPHS1* and *NPHS2* in four individuals from three different families with congenital FSGS. One patient showed a homozygous *NPHS1* mutation and only one heterozygous *NPHS2* mutation (R229Q). Three patients (from two families) showed only one heterozygous *NPHS1* mutation and a homozygous *NPHS2* mutation. Caridi et al. [27] reported one patient with only one heterozygous *NPHS1* mutation and only one heterozygous *NPHS2* mutation (R229Q). To further evaluate these hypotheses of a digenic effect for mutations in *NPHS1* and *NPHS2,* we performed mutational analysis of *NPHS1* in a cohort of 74 patients in whom we had previously performed analysis of *NPHS2* [8]. We describe here 5 patients with a "triallelic hit", 4 patients (F435, F451, F1296, A140) with two recessive *NPHS1* mutations and only one heterozygous *NPHS2* mutation (Table 1, subgroup B), and one patient (F1268) with only one heterozygous *NPHS1* mutation and two homozygous *NPHS2* mutations (Table 1, subgroup A). In all 4 patients of subgroup B, the single heterozygous mutation in the *NPHS2* gene was R229Q. This mutation has previously been described as a polymorphism, since it was found in 3% of all chromosomes in 100 healthy controls [7]. A functional relevance had been claimed for R229Q. The codon is highly conserved in evolution, the amino acid exchange is non-conservative, and it segregated with the disease in certain families. The encoded peptide is altered and the interaction with nephrin in nephrin-binding assays diminished [25]. However, our group and others recently detected R229Q in patients with SRNS, SSNS, and in healthy controls [8, 27]. In this study, in the 4 patients bearing this sequence variant as the third altered allele, the two recessive *NPHS1* mutations were sufficient to explain their CNS phenotype. As far as clinical data were available, no significant clinical pleiotropic difference was noticed between these 4 patients and CNS patients with two recessive *NPHS1* mutations and absence of *NPHS2* mutation (Table 1, subgroup B and C, respectively).

In F1268 we found only one heterozygous *NPHS1* mutation and two homozygous *NPHS2* mutations (Table 1, subgroup A). The two homozygous *NPHS2* mutations found in this patient have both been previously described [7, 8]: an insertion of T at position 460–467 inducing a frameshift resulting in a stop codon at V165X and a G709C tranversion leading to a conservative amino acid exchange E237Q (Table 1, subgroup A). To date the second sequence variant has only been found heterozygously by our group in 1 SRNS and 1 SSNS patient [8]. Recently, we detected this sequence variant heterozygously paired in *cis* with a heterozygous splice site mutation and in *trans* with a heterozygous deletion in a SRNS patient (Mucha et al., unpublished data). These findings support the suspicion that E237Q might be a rare polymorphism, even though it was absent from 80 healthy controls.

The *NPHS1* mutation G2971C that leads to a conservative amino acid exchange V991L was found heterozygously in F1268. This amino acid exchange is conservative and the codon is not conserved in evolution. We could not find this mutation in 196 chromosomes of 98 healthy control individuals. However, since this family is from the Arab country of Oman, it might still represent a rare polymorphism in the Arab population, not detected in healthy controls from central Europe. Since both the *NPHS2* variant E237Q and the *NPHS1* variant V991L probably represented innocuous polymorphisms, the patient's phenotype most likely is due to the homozygous *NPHS2* mutation V165X (Table 1, subgroup A). This is consistent with the finding that the clinical data available for this patient are similar to clinical data from patients with two recessive *NPHS2* mutations and no *NPHS1* mutation (Table 1, subgroup A; [8]): Age at onset was 1.6 years compared with 2.4 years; initial symptoms were acute edema and fever. Steroid therapy was started recently. No biopsy has been performed to date.

Caridi et al. [27] reported one patient with one heterozygous *NPHS1* mutation only (amino acid exchange A907T) and R229Q in *NPHS2*. This patient has an age at onset of proteinuria of 1.4 years, is steroid sensitive, and has FSGS histology. However, the patient's healthy mother showed the same molecular findings.

Koziell et al. [15] reported co-existence of *NPHS1* and *NPHS2* mutations in four patients from three families. The third mutation was suspected to modify the histological phenotype from CNS of the Finnish type (CNF) to congenital FSGS, which was observed in all 4 patients. We report 5 patients with combined *NPHS1* and *NPHS2* mutations. Biopsy showed MCNS in 1 of 5 patients, DMS in 1 of 5 patients, and was not performed in 3 of 5 patients (Table 1, subgroup A and subgroup B). Congenital FSGS was not reported in any of our patients, so we cannot compare these findings.

We further examined another 12 CNS patients without *NPHS2* mutation. In 11 of 12 patients two recessive *NPHS1* mutations were found (Table 1, subgroup C), explaining their CNS phenotype. Two patients (F475 and F1328) also had affected siblings and shared the genotype with them. The clinical expression of the disease in these siblings was indistinguishable (Table 1, subgroup C).

In family F1328 we report 2 affected individuals with compound heterozygous *NPHS1* mutations and an unusually mild phenotype for the disease, characterized by absence of ESRD even at 24.0 and 20.0 years of age (Table 1, subgroup C). The mutations are a heterozygous C385T transition leading to the conservative amino acid exchange L130F, and a heterozygous G1868T transversion leading to the non-conservative amino acid exchange C623F. None of our other patients showed these mutations. L130F is novel; a heterozygous C623F mutation was described previously [23]. Other cases with a mild phenotype have been described with *NPHS1* mutations. Remission of proteinuria in cases with characteristic CNF histology has been reported [28]. Koziell et al. [15] described a mild phenotype in Maltese patients with ho-

mozygous R1160X, a founder mutation in the Maltese population. Patrakka et al. [22] reported clinical features resulting from different *NPHS1* mutations. They describe a milder phenotype in one patient with Fin major and a missense mutation compared with patients with Fin major and/or Fin minor mutations only [22]. F1328 II-1 is a 24-year-old patient with NS from birth. He is steroid resistant and his renal biopsy (obtained at the age of 7.8 years) showed mild FSGS. To date his renal function is normal and he does not need treatment. His sister F1328 II-3, who shares the same genotype, also exhibits a very similar phenotype (NS, normal renal function, no treatment). Her renal histology, however, differs and was without pathological findings at the age of 19.5 years (by light microscopy, immunohistochemistry, and electron microscopy). These findings emphasize that there can be rare cases of unusually mild phenotypes in patients with *NPHS1* mutations.

In the group of SRNS patients with two recessive *NPHS* 2 mutations (Table 2, group I), 11 of 48 (23%) patients showed CNS. This confirms the finding that CNS, which usually results from recessive mutations in *NPHS1*, can also result from *NPHS2* mutations [8, 15], and supports the genetic heterogeneity of CNS. The percentage of CNS patients with *NPHS2* mutations is still surprisingly high. Within the group of 27 patients with CNS (Table 2 11 patients of group I, 4 patients of group II, and 12 patients of group III), we found in 15 (55%) patients two recessive *NPHS1* mutations, and in 11 of 27 (41%) patients two recessive *NPHS2* mutations. No mutation was found in 1 of 27 (4%) patients with CNS.

When comparing phenotypes between these two CNS groups (patients with two recessive *NPHS1* mutations versus patients with two recessive *NPHS2* mutations), we notice especially differences in the biopsy results. Of the CNS patients with two recessive *NPHS2* mutations, 7 of 14 (50%) showed FSGS (2/14 MCNS, 1/14 CNF, 1/14 MP, 3/14 not performed) [8]. This confirms the previous findings, since *NPHS2* has been described as causative for SRNS with FSGS [6]. Karle et al. [7] found FSGS in 75% of *NPHS2* patients. Biopsy findings in the 15 CNS patients with two recessive *NPHS1* mutations (Table 1, subgroup B and subgroup C) were surprisingly diverse: only 2 of 15 results showed the histological picture of CNF [in 1 case the biopsy report could not exclude CNF (F435)]. In 3 of 15 patients the biopsy revealed MCNS, which is unusual for *NPHS1* patients. One patient showed mild FSGS, but the biopsy was performed at the age of 7.8 years. DMS was found in 2 of 15 patients. In 5 of 15 patients no biopsy was performed, and in 2 of 15 results were not available. In CNS patients with two recessive *NPHS2* mutations, 6 of 14 (43%) developed ESRD at a median age of 6.0 years and all 6 (43%) had kidney transplants at a median age of 7.2 years [8]. In CNS patients with two recessive *NPHS1* mutations, 5 of 15 (33%) developed ESRD at a median age of 3.0 years and all 5 (33%) had kidney transplants at a median age of 4.0 years (Table 1, subgroup B and subgroup C).

In addition to the 19 *NPHS1* mutations in Table 1, we identified in 8 patients the known sequence variant G1223A (R408Q) heterozygously. Lenkkeri et al. [23] considered R408Q a mutation, even though it was found heterozygously in 4 of 30 healthy control individuals, and in a Finnish patient, in addition to 2 other heterozygous mutations. We detected this sequence variant heterozygously in 5 of 49 (10%) patients of group I, 1 of 14 (7%) patients of group II, 2 of 14 (14%) patients of group III (F228, F1017) and 10 of 83 (12%) healthy controls, and therefore consider it an innocuous polymorphism.

In conclusion, in this study we did not find any evidence for di-genic inheritance of *NPHS1* and *NPHS2* mutations as a "tri-allelic hit" that would result in phenotypic modifications. Compared with previously published data based on a limited number of patients, the frequency of patients carrying combined mutations in *NPHS1* and *NPHS2* is rather low. We report 4 patients with two recessive *NPHS1* mutations in addition to the heterozygous *NPHS2* sequence variant R229Q. The phenotype of our 4 patients (Table 1, subgroup B) was not altered by the presence of R229Q. A striking difference in the phenotype of these patients compared with the phenotype of patients with two recessive *NPHS1* mutations without *NPHS2* mutation could not be observed.

In 1 patient only one heterozygous *NPHS1* mutation and two recessive *NPHS2* mutations were found (Table 1, subgroup A), as reported by Koziell et al. [15] for 3 patients from two families. The phenotype in our patient does not differ from other patients with two recessive *NPHS2* mutations and without mutation in *NPHS1*.

A patient with only one heterozygous *NPHS1* mutation and only one heterozygous *NPHS2* mutation, as described earlier [27], has not been identified in our study. In the limited number of 5 patients, our data does not suggest that patients with combined mutations in *NPHS1* and *NPHS2* show any genotype/phenotype correlation.

Acknowledgements We thank A. Imm for excellent technical assistance. A.F. and F.H. were supported by a grant from the Fritz-Thyssen-Stiftung. F.H. was supported by a grant from the George O'Brien Renal Center (2P50DK039255–16-P2).

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