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Co-Inheritance of Functional Podocin Variants with Heterozygous Collagen IV Mutations Predisposes to Renal Failure

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Key Words

Thin basement membrane nephropathy · Focal segmental glomerulosclerosis · Collagen type IV · Genetic polymorphism · Podocyte markers · Glomerular basement membrane

Abstract

Background/Aims: A subset of patients who present with proteinuria and are diagnosed with focal segmental glomerulosclerosis (FSGS) have inherited heterozygous *COL4A3/A4* mutations and are also diagnosed with thin basement membrane nephropathy (TBMN-OMIM: 141200). Two studies showed that co-inheritance of *NPHS2*-p.Arg229Gln, a podocin variant, may increase the risk for proteinuria and renal function decline. ***Methods:*** We hypothesized that additional podocin variants may exert a similar effect. We studied genetically a well-characterized Cypriot TBMN patient cohort by re-sequencing the *NPHS2* coding region. We also performed functional studies in cell culture experiments, investigating the interaction of podocin variants with itself and with nephrin. ***Results:*** Potentially disease-modifying podocin variants were searched for by analyzing *NPHS2* in 35 'severe' TBMN patients. One non-synonymous variant, p.Glu237Gln, was detected. Both variants, p.Arg229Gln and p.Glu237Gln, were tested in a larger cohort of 122 TBMN patients, who were categorized as 'mild' or 'severe' based on the presence of microscopic hematuria alone or combined with chronic renal failure and/or proteinuria. Seven 'severe' patients carried either of the 2 variants; none was present in the 'mild' patients ($p = 0.05$, Pearson χ^2). The 7 carriers belong in 2 families segregating mutation *COL4A3*-p.Gly1334Glu. Inheritance of the wild-type (WT) and mutant alleles correlated with the phenotype (combined concordance probability 0.003). Immunofluorescence (IF) experiments after dual co-transfection of WT and mutant podocin suggested altered co-localization of mutant homodimers. IF experiments after co-transfection of WT podocin and nephrin showed normal membrane localization, while both podocin variants interfered with normal trafficking, demonstrating perinuclear staining. Immunoprecipitation experiments showed stronger binding of mutant podocin to WT podocin or nephrin. ***Conclusion:*** The results support the hypothesis that certain hypomorphic podocin variants may act as adverse genetic modifiers when co-inherited with *COL4A3/A4* mutations, thus predisposing to FSGS and severe kidney function decline.

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Introduction

A percentage of about 40% of patients with thin basement membrane nephropathy (TBMN-OMIM: 141200) carries heterozygous mutations in the *COL4A3/A4* genes that affect the structure of the glomerular basement membrane (GBM) [1, 2]. Homozygous or compound heterozygous mutations in the same genes cause classic autosomal recessive Alport syndrome [3–9]. *COL4A3/A4/A5* genes encode the 3 chains of collagen IV, which is the major structural component of GBM [10]. GBM is considered a very significant part of the glomerular filtration barrier; the other 2 parts are the fenestrated endothelium and the podocytes. Podocytes form long, interdigitated foot processes that enwrap the glomerular capillaries. The foot processes are connected by a thin structure termed the glomerular slit diaphragm (SD), forming a significant barrier impeding protein leakage [11].

TBMN was long considered a benign renal condition, invariably presenting with isolated microscopic hematuria and normal kidney function [3]. Older scarce reports had suggested that occasionally TBMN can be progressive, leading to chronic renal failure (CRF) of variable degree. In more recent publications, we and others showed that a substantial percentage of *COL4A3/A4* heterozygous mutation carriers may develop focal and segmental glomerulosclerosis (FSGS) [12–14] or even develop ultrastructural GBM histology of alternate thinning/thickening and splitting, which is pathognomonic of Alport syndrome; the only difference being that this time these carriers follow the autosomal dominant mode of inheritance [15]. Overall, our data showed that in a large Greek-Cypriot cohort, more than 50% of TBMN patients with known *COL4A3/A4* heterozygous mutations develop clinically significant proteinuria and CRF due to late-onset FSGS, manifesting after the age of 30 years. Another equally important aspect is that in a Greek-Cypriot cohort of 230 patients, about 30% of the patients reached end-stage renal disease (ESRD) by the age of 70 years [16].

Environmental and genetic factors have been invoked in explaining the progressive nature of TBMN in a subset of patients. Two different studies have demonstrated that variant p.Arg229Gln in podocin, encoded by the *NPHS2* gene, predisposes TBMN patients to proteinuria and renal failure [17, 18]. Pathogenic podocin mutations are responsible for the steroid-resistant nephrotic syndrome (SRNS-OMIM: 600995), which follows the autosomal recessive mode of inheritance. Podocin is a membrane-integral protein belonging to the stomatin family, and in the kidney it is exclusively localized at the cytoplasmic face of the SD, which bridges podocyte foot processes [19–21]. Saleem et al. [22] showed that it co-localizes with nephrin and cytoskeletal actin while they provided evidence for its role in the foot process formation. Disease-causing mutations in the *NPHS2* gene in patients with SRNS were shown to not only cause misfolding of podocin and alter its localization, but they also altered the trafficking of normal nephrin to the plasma membrane [23, 24]. Moreover, several domains of podocin have been demonstrated to facilitate specific functions of this protein, such as oligomerization in lipid raft microdomains, membrane association and interaction with other SD components, including nephrin and CD2AP [24, 25].

Here, we used a previously described TBMN cohort [18] that was enriched with enrolment of additional patients and we focused on families that segregate TBMN due to a primary founder mutation *COL4A3*-p.Gly1334Glu. We also searched for additional putative variants in the *NPHS2* gene that might account for the intrafamilial variability by conferring higher risk. We hypothesized that other non-synonymous hypomorphic variants in the same gene, besides *NPHS2*-p.Arg229Gln, could act as genetic modifiers [26] and predispose to an adverse disease outcome in TBMN during aging. We identified a second variant, p.Glu237Gln, and both were tested in cell culture experiments for their ability to impact podocin localization and cross-interaction with nephrin.

Results

Genetics Studies

We were interested in searching for putative functional variants in the *NPHS2* gene that might act as adverse genetic modifiers of TBMN. This hypothesis was based on previous results according to which a known variant, p.Arg229Gln was shown to predispose such patients to proteinuria and severe CRF. We re-sequenced the *NPHS2* gene of 35 'severe' TBMN patients and identified a previously described rare non-synonymous variant, p.Glu237Gln in exon 5, in 3 patients. We then genotyped 122 patients of our TBMN cohort by DNA re-sequencing of exon 5 (see table 1 for description of the cohort). We found, in total, 3 p.Glu237Gln and 4 p.Arg229Gln heterozygous patients among the 82 'severe' patients and none among the 40 'mild' patients. Genotypic association analysis by Pearson Chi-square gave a p value of 0.05 (table 2).

The 3 severely affected patients carrying variant p.Glu237Gln belonged to the CY5304 family and the other 4 carrying variant p.Arg229Gln belonged to the

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Table 1. Description of the cohort of TBMN patients ('mild' patients born before January 1963) under study

Patients	Origin	n	Mild				Severe			
			n (%)	age, mean ± SD	females, n (%)	with ESRD, n (%)	n (%)	age, mean ± SD	females, n (%)	with ESRD, n (%)
Heterozygous *COL4A3* or *COL4A4* mutation carriers	Cyprus	122	40 (33)	60.9±11.1	28 (70)	0	82 (67)	59.6±13.1	34 (41)	25 (30)

Age difference (mild vs. severe) is not significant (p = 0.583). Gender difference (mild vs. severe) is significant (p = 0.003).

Table 2. Combined frequencies and statistics of *NPHS2*-p.Arg229Gln and *NPHS2*-p.Glu237Gln variants, among patients with thin basement membrane nephropathy and according to disease severity

Cohort	n	Genotype counts			Genotype frequency, %			p values
		229RR/237EE	229RQ/237EQ	229QQ/237QQ	229RR/237EE	229RQ/237EQ	229QQ/237QQ	
TBMN-mild	40	40	0	0	100.0	0	0	0.09 (2-sited Fisher's exact test) 0.08 (Bernard test) 0.05 (Pearson χ^2)
TBMN-severe	82	75	7	0	91.5	8.5	0	

CY5376 family. Both families co-segregated the same *COL4A3*-p.Gly1334Glu as the primary TBMN mutation with one of the 2 *NPHS2* variants. In these 2 families, CY5304 and CY5376, we had DNA for 7 and 11 TBMN patients respectively. Interestingly, in the CY5304 family, we found an *NPHS2*-p.237Gln homozygous individual (III-4), negative for the *COL4A3*-p.Gly1334Glu mutation, aged 81, with no known renal phenotype (fig. 1). The 3 'severe' patients of this family were heterozygous carriers of *NPHS2*-p.Glu237Gln (indicated with a cross (+) symbol in fig. 1), while the 4 'mild' ones were negative for the *NPHS2*-p.Glu237Gln variant. This shows a positive concordance of the p.Glu237Gln with a severe phenotype, when on the background of *COL4A3*-p.Gly1334Glu. The probability for this to happen is fairly low. According to the genotyping of subjects in generation III (no DNA samples are available from generation II), both parents II-1 and II-2 were obligate heterozygous for the *NPHS2*-p.Glu237Gln, whereas in the case of spouses/parents II-3 and II-4, most probably only 1 was positive (heterozygous) for this variant (fig. 1). Here we took into account 3 severely affected patients as well as 2 mildly affected patients for calculating the probability for having concordance of a severe phenotype segregating by chance with the p.Glu237Gln variant of podocin, on the background of TBMN. The other 2 mildly affected patients IV-13, IV-15 were excluded, as their mildly affected mother was found to have the homozygous wild-type for the *NPHS2* gene. Thus, the probability for the *NPHS2*-p.Glu237Gln to segregate by chance in the 5 individuals with TBMN, is $(0.75) \times (0.5)^4 = 0.05$. The probability of 0.75 is attributed to patient III-1, whose parents were obligate carriers. Patient IV-10, who is a carrier of a known collagen mutation and one of the podocin variants, had a biopsy proven FSGS on the background of TBMN.

In the CY5376 family (fig. 2), we observed the segregation of *NPHS2*-p.Arg229Gln in patients who satisfied the criteria for a severe phenotype (II-2, II-4, II-9, III-13). Mildly affected subjects were not included in this study because of their young age, thereby not satisfying the set criteria (see Materials and Methods). Again, according to the genotyping data in generation II (fig. 2) and based on the rarity of the p.Arg229Gln allele, we assume that only one of the 2 parents I-1 and I-2 is heterozygous for this

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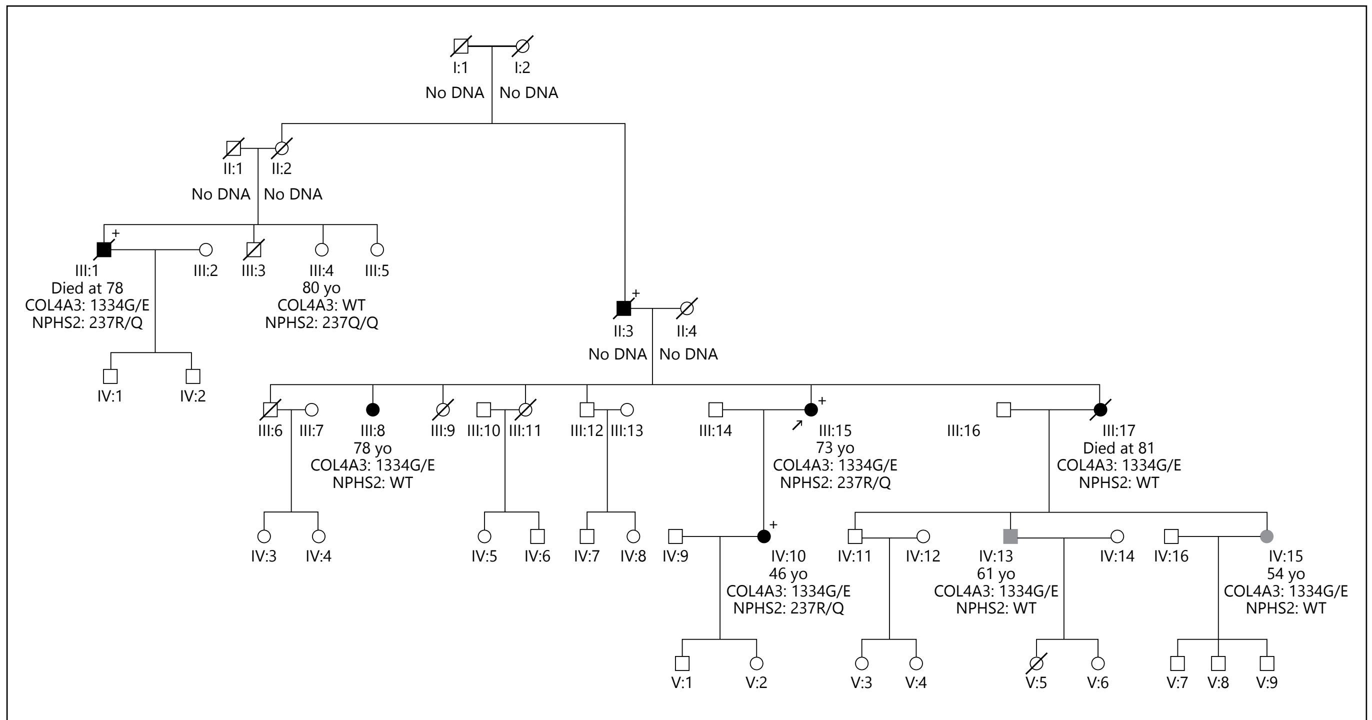

Fig. 1. The CY5304 family, where *NPHS2* variant p.Glu237Gln was found to segregate with the severe phenotypes. Black symbols show the thin basement membrane nephropathy patients, heterozygous for the founder mutation *COL4A3*-p.Gly1334Glu (COL4A3: 1334 G/E). Patients with a cross (+) symbol have a severe phenotype. The biopsy of patient IV-10 shows FSGS on the background of thin glomerular basement membranes. Patients IV-13, IV-15 are depicted with filled grey symbols, because they were excluded from the cohort under study, as their mildly affected mother is homozygous for the wild-type *NPHS2* gene. WT: normal; COL4A3: 1334G/E, heterozygous for *COL4A3*-p.Gly1334Glu; NPHS2: 229R/Q, heterozygous for *NPHS2*-p.Arg229Gln; NPHS2: 237Q/Q, homozygous for *NPHS2*-p.237Gln.

variant. Hence, the probability of *NPHS2*-p.Arg229Gln to segregate by chance in the 4 patients is $(0.5)^4 = 0.06$ (nearly significant). Studying more carefully the clinical record of individual II-7, we found that the patient also had vesicoureteral reflux (VUR). This might explain his progression to ESRD at age 55 years, without having inherited *NPHS2*-p.Arg229Gln. We found additional evidence that VUR can contribute to TBMN severity when we realized that his nephew, individual III-13, also had VUR and he reached ESRD at the young age of 37 years. This patient had reached ESRD at the youngest age among all 248 Greek-Cypriot patients with TBMN for whom we have records. We attribute this to the simultaneous inheritance of both the *NPHS2*-p.Arg229Gln variant and VUR.

Taken together, the inheritance results in the 2 families show that the combined probability for a severe TBMN phenotype to co-segregate with the less frequent p.Arg229Gln or p.Glu237Gln variants by chance is $0.05 \times 0.06 = 0.003$. Additional clinical data for all the 7 'severe' patients carrying a modifier variant can be found in table 3.

Immunofluorescence Experiments

In order to examine whether the podocin variants identified in patients have any effect on protein localization, we co-expressed the mutants with the wild-type (WT) protein in the same cells and studied dually expressing cells. Specifically, cells were dually transfected with either genes expressing WT HA-tagged podocin and WT FLAG-tagged podocin or expressing the WT HA-tagged protein and FLAG-tagged mutant proteins. Confocal microscopy analysis revealed somewhat altered localization of both variants as compared to the WT protein. Specifically, while the wild-type FLAG- and HA-tagged podocins co-localized fully in the plasma membrane, this was not the case with the p.Arg229Gln or p.Glu237Gln, where both variants demonstrated less uniform imperfect co-localization with the WT proteins, with more prominent perinuclear staining. However, the difference was inconclusive as judged by immunofluorescence, most probably because of a subtle effect (not shown).

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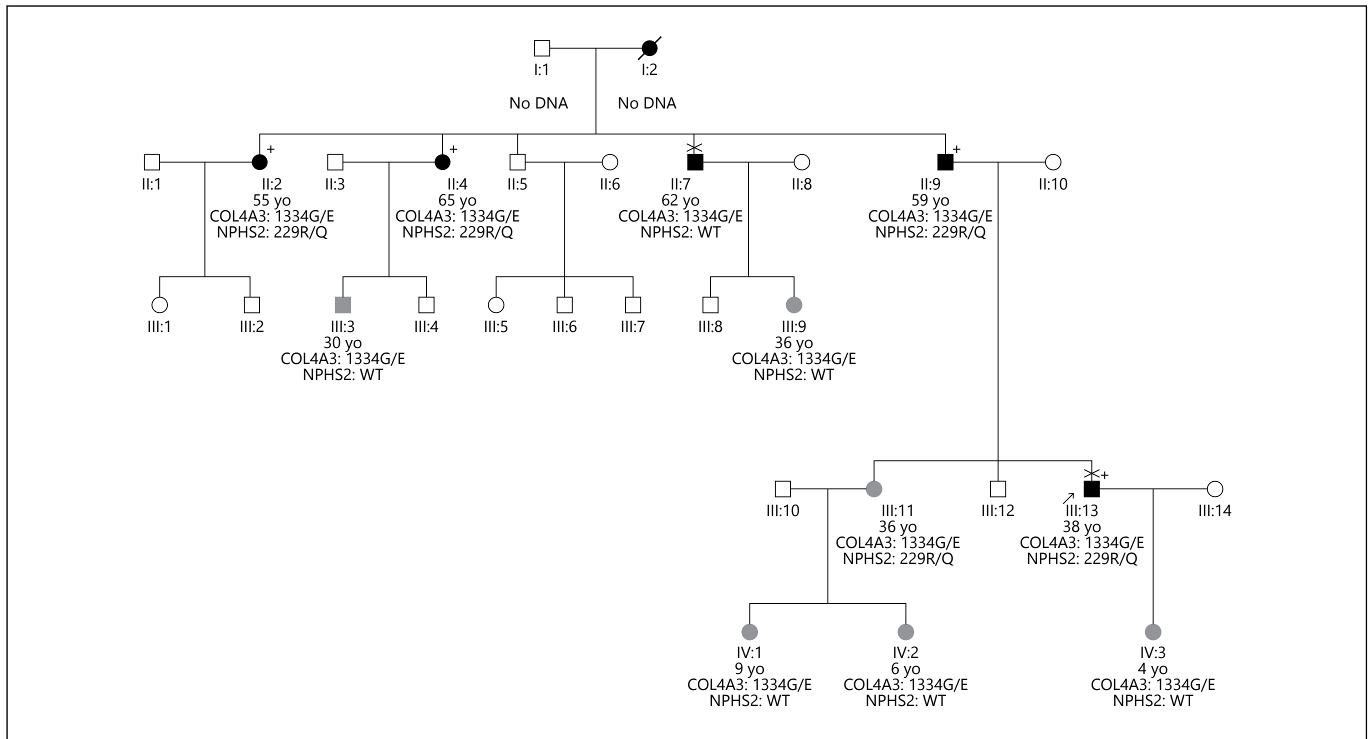

Fig. 2. The CY5376 family where *NPHS2* variant p.Arg229Gln was found to segregate with the severe phenotype. Black symbols show the thin basement membrane nephropathy patients, heterozygous for the founder mutation *COL4A3*-p.Gly1334Glu (COL4A3: 1334G/E). Patients with a cross (+) symbol have a severe phenotype. Patient II-7 also has a severe phenotype, which is attributed to his co-occurrence of thin basement membrane nephropathy and VUR. He was not used for calculation of the concordance probability. Some other patients are heterozygous for the risk variant p.Arg229Gln and have mild disease but were not accounted among the cohort of the 122 patients because they are very young and consequently do not satisfy the set criteria (see Materials and Methods). Patients who were excluded from the cohort under study due to young age, are depicted with filled grey symbols. The 2 patients marked with an (×) symbol had exhibited VUR in childhood. WT: normal; COL4A3: 1334G/E, heterozygous for *COL4A3*-p.Gly1334Gln; NPHS2: 229R/Q, heterozygous for *NPHS2*-p.Arg229Gln.

Table 3. Clinical information for the 7 'severe' patients carrying a heterozygous mutation in *COL4A3* and a modifier in the *NPHS2* gene

Family/patient/gender	Mutations	Age at ESRD	Biopsy	Other*	Age by 2013	Age of death
CY5304/III-1/male	*COL4A3*-p.Gly1334Glu/ *NPHS2*-p.Glu237Gln	78	ND			78
CY5304/III-15/female	*COL4A3*-p.Gly1334Glu/ *NPHS2*-p.Glu237Gln	67	ND		73	
CY5304/IV-10/female	*COL4A3*-p.Gly1334Glu/ *NPHS2*-p.Glu237Gln		TBMN-FSGS	Scr: 0.93 mg/dl proteinuria: 700 mg/24 h	46	
CY5376/II-2/female	*COL4A3*-p.Gly1334Glu/ *NPHS2*-p.Arg229Gln		ND	Scr: 1.70 mg/dl proteinuria: 1,200 mg/24 h	55	
CY5376/II-4/female	*COL4A3*-p.Gly1334Glu/ *NPHS2*-p.Arg229Gln		ND	Scr: 1.45 mg/dl proteinuria: 600 mg/24 h	65	
CY5376/II-9/male	*COL4A3*-p.Gly1334Glu/ *NPHS2*-p.Arg229Gln		ND	Scr: 1.40 mg/dl proteinuria (not measured recently)	59	
CY5376/III-13/male	*COL4A3*-p.Gly1334Glu/ *NPHS2*-p.Arg229Gln	37	ND	vesicoureteral reflux since childhood	38	

* Biochemical data by 2013. ND = Not done.

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We next tested whether the podocin variants identified had an effect on nephrin, a crucial component of the SD and a known partner of podocin. For these experiments, undifferentiated human podocytes were transiently co-transfected with podocin constructs expressing the WT and mutant forms of podocin and with a construct expressing nephrin. When WT podocin was expressed with WT nephrin, the proteins demonstrated intense diffuse co-localization to the plasma membrane, contrary to p.Arg229Gln or p.Glu237Gln, where both variants interfered with the normal trafficking of nephrin and exhibited a dominant negative effect in altering its localization to a perinuclear fashion (fig. 3).

Co-Immunoprecipitation Results

On the basis of the fact that podocin variants demonstrated somewhat altered cellular localization, we next tested whether podocin homodimerization was altered in the presence of the variants. We carried out co-immunoprecipitation experiments using HEK293T cells transiently co-transfected with either the WT podocin and/or the mutant podocin constructs tagged with a different epitope. An important observation made was that the results showed that WT podocin interacted significantly strongly with the variants as compared to the way WT protein interacted (fig. 4). Specifically, WT podocin had 209% stronger binding to p.Arg229Gln podocin (n = 5, p = 0.048) and 244% to p.Glu237Gln (n = 5, p = 0.035) (unpaired two-tailed t tests).

Since the co-expression of mutant podocin also alters nephrin localization, we next examined the interaction properties of the 2 proteins in the presence of the variants. HEK293T cells were transiently co-transfected with the cDNAs of the WT nephrin and the WT or mutant podocin. In these immunoprecipitation assays, we observed less nephrin interacting with WT podocin than with either of the 2 mutants (fig. 5). The normalized intensity of nephrin immunoprecipitated by p. Arg229Gln podocin demonstrated a 149% increase, compared to WT podocin (n = 4 unpaired comparisons, p = 0.049 in an unpaired two-tailed t test), while the normalized intensity of nephrin immunoprecipitated by p.Glu237Gln podocin showed a 297% increase compared to WT podocin (n = 4 unpaired comparisons, p = 0.0074 in an unpaired two-tailed t test). In the absence of podocin, nephrin did not immunoprecipitate, while the western blot analysis indicated that equivalent amounts of podocin were immunoprecipitated in each reaction.

Discussion

The great intra- and inter-familial phenotypic heterogeneity that we observed among our patients with TBMN prompted us to hypothesize a role for putative modifier genes. Extended founder effects found in Cyprus for 2 mutations in the *COL4A3* gene can greatly help in identifying modifier variants due to less prominent genetic background 'noise'. Previously published data [17, 18] encouraged us to proceed in direct re-sequencing of the entire coding region of the *NPHS2* gene in 'severe' patients, with the hope to identify more candidate modifier variants. We found 7 patients with severe phenotype that carried variant p.Arg229Gln or p.Glu237Gln, while none of the mildly affected patients had inherited any non-synonymous variant of obvious significance (p = 0.05).

Information for *NPHS2*-p.Arg229Gln in heterozygosity is continuously increasing in bibliography, with ambiguous findings. It is well established that p.Arg229Gln is a frequent cause of nephrotic syndrome when found in compound heterozygosity with other *NPHS2* mutations [26–31]. However, its putative significance when in heterozygosity is still under investigation. Pereira et al. [32] reported that p.Arg229Gln is responsible for micro-albuminuria in the general population. In contrary, Tonna et al. [33] found that p.Arg229Gln does not alter the risk of proteinuria in the general population. Köttgen et al. [34] did not corroborate any significant association between p.Arg229Gln and GFR in either white or black individuals. Based on a very detailed review and meta-analysis article by Franceschini et al. [35], the p.Arg229Gln variant gives a non-significant increased risk for FSGS by 20 to 70% in populations of European descent. Jungraithmayr et al. [36] refer to a possible association of p.Arg229Gln with kidney transplant rejection.

In Cyprus, we found a frequency of 2% for the p.Arg229Gln allele [18] and a frequency of 2.1% for the p.Glu237Gln allele (4 heterozygous in 96 DNA samples of the general population studied here). Contrary to what is available for variant p.Arg229Gln, bibliographical information about p.Glu237Gln is scanty. Several investigators find this variant in heterozygosity in a few sporadic cases of nephrotic syndrome and FSGS, stating that its significance is unknown [33, 37, 38]. Ruf et al. [38] did not find this variant in controls, whereas Weber et al. [37] found a frequency of 0.3% in the general population, for the rare p.Glu237Gln allele.

The family segregation studies presented here provide evidence that these variants can behave as modifiers for TBMN, perhaps through the progression and the devel-

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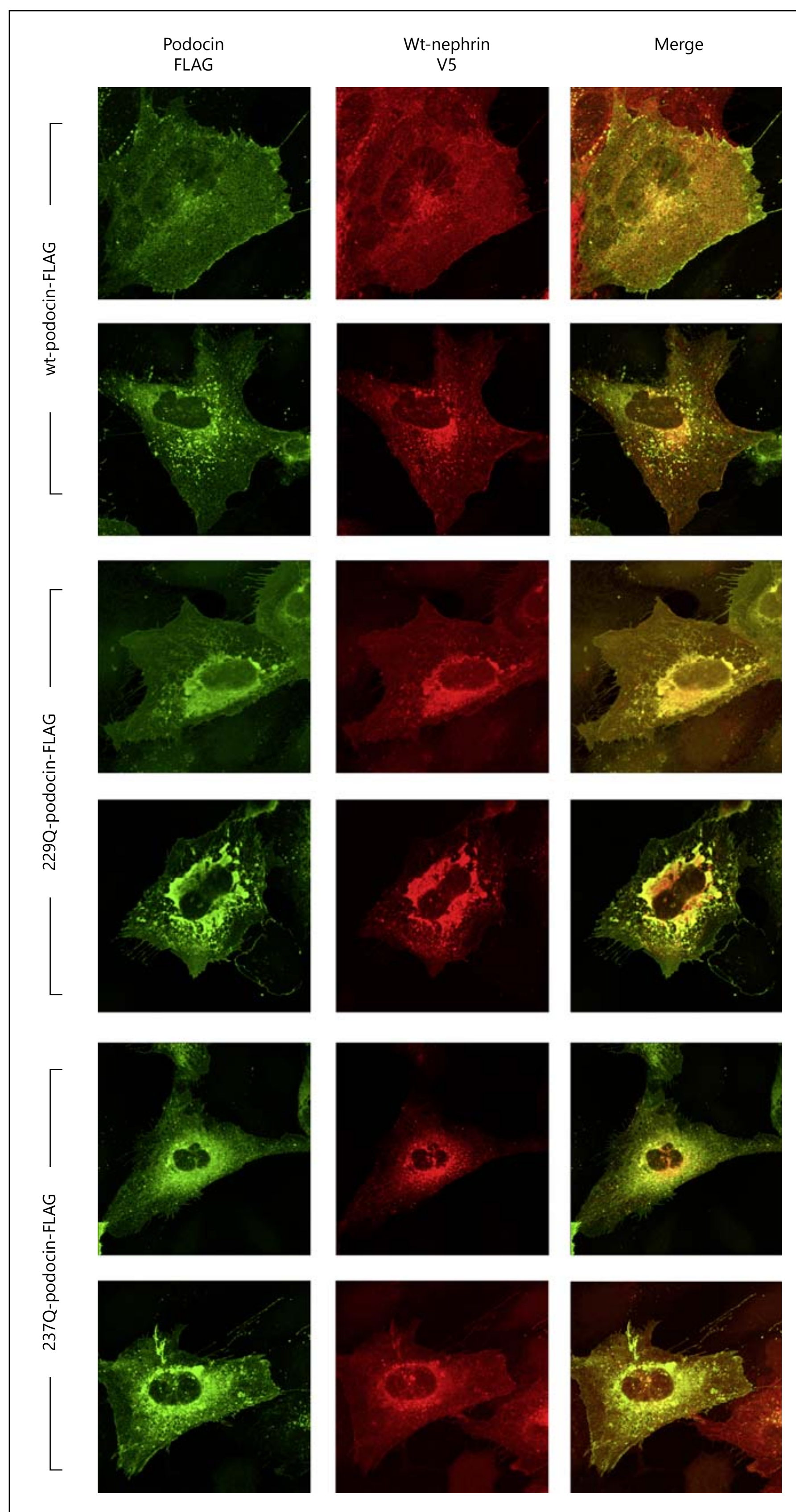

Fig. 3. Double-labelling immunofluorescence of WT nephrin (NPHS1, red) and WT or missense mutated podocin (NPHS2, green). Human podocyte cell line was transiently double transfected with the WT nephrin and with either wild-type or each missense mutated podocin cDNAs, followed by immunostaining with anti-V5 (red) antibody and anti-FLAG (green) antibody (colors refer to the online version only). WT nephrin co-localizes with wild-type podocin, both properly localizing to the plasma membrane, contrary to podocin 229Gln (229Q) or podocin 237Gln (237Q), where both variants showed to interfere to the normal trafficking of both proteins, demonstrating a more prominent perinuclear staining. Note: shown are the 3 experimental settings, shown in duplicates, 2 in the upper, 2 in the middle and 2 in the lower panels.

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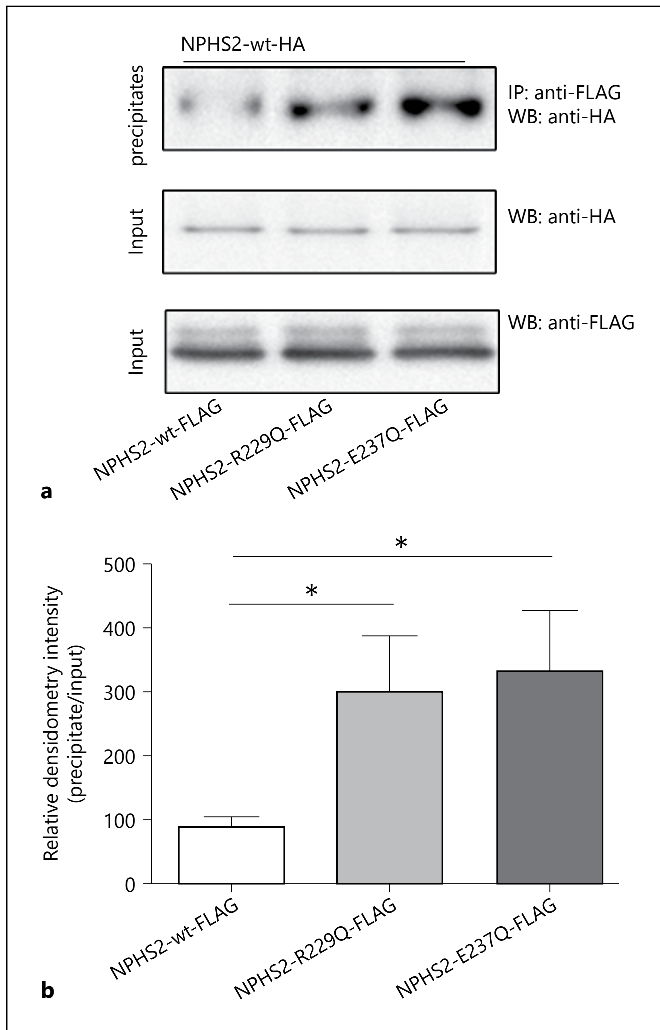

Fig. 4. a Podocin homodimerization experiments: Podocin co-precipitates with both variants (p.Arg229Gln and p.Glu237Gln). FLAG- and HA-tagged proteins were expressed in HEK293T cells and precipitated with anti-FLAG antibody as indicated. Western blot analysis was performed with HA and FLAG specific antibodies. Expression levels of HA-tagged WT podocin and FLAG-tagged variant constructs in the lysates are shown below. **b** Densitometry of the p.Arg229Gln and p.Glu237Gln bands from repeated experiments (n = 5). Intensity is given as percentage of wild-type intensity, which is set by definition at 100% (shown are mean values, with bars indicating SEM). As shown, both podocin variants demonstrate significantly increased homodimerization with the WT protein. With p.Arg229Gln there is 209% increase (p = 0.048) and with p.Glu237Gln there is 244% increase (p = 0.035).

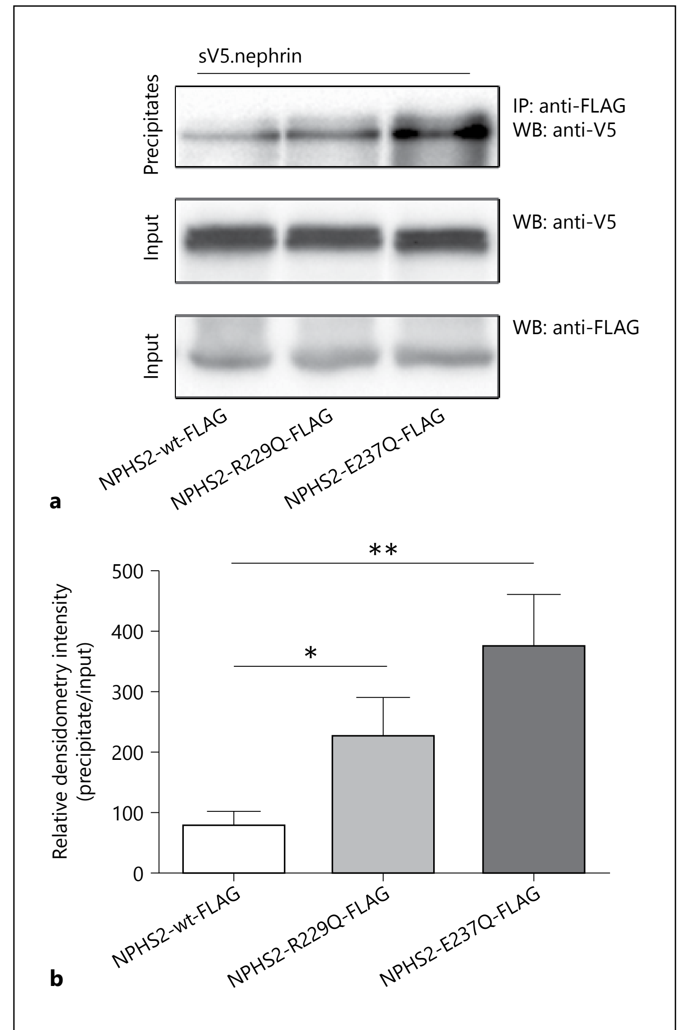

Fig. 5. a Podocin and nephrin interaction experiments. Nephrin co-precipitates with both podocin variants (p.Arg229Gln and p.Glu237Gln). FLAG- and V5-tagged proteins were expressed in HEK293T cells and precipitated with anti-FLAG antibody as indicated. Western blot analysis was performed with a V5 and FLAG specific antibodies. Expression levels of FLAG podocin and V5 nephrin constructs in the lysates are shown below. **b** Densitometry of the p.Arg229Gln and p.Glu237Gln bands from repeated experiments (n = 4). Intensity is given as percentage of wild-type intensity, which is set by definition at 100% (shown are mean values, with bars indicating SEM). As shown, both podocin variants demonstrate significantly increased interaction with WT nephrin. With p.Arg229Gln there is 149% increase (p = 0.049) and with p. Glu237Gln there is 297% increase (p = 0.0074).

opment of histological FSGS. Perhaps a documented case is individual IV-10 in family CY5304 (fig. 1). The combined probability for these observations to have occurred by chance is very small, that is, 0.003. Additionally, to our knowledge, we described the first individual who is homozygous for the p.Glu237Gln variant. Of importance is the fact that homozygosity for this variant alone did not seem to cause a conspicuous phenotype.

Tsukaguchi et al. [39] proved that p.Arg229Gln decreases binding of nephrin to podocin and very recently

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Tory et al. [31] found that disease-associated 3′ mutations of podocin exerted a dominant-negative effect on p.Arg229Gln podocin but behaved as recessive alleles when associated with WT podocin. No corresponding functional studies exist for variant p.Glu237Gln. Both variants concern evolutionarily conserved amino acid residues, suggesting a functional significance. We hypothesize that the presence of these variants may adversely affect the cross-interactions of SD proteins, thereby predisposing to mechanical instability and deterioration of the filtration barrier as a long-term effect. Here, in our hands, both variants appeared to increase both podocin homodimerization as well as binding of podocin with nephrin (fig. 4, 5). Additionally, we showed by immunofluorescence microscopy that p.Glu237Gln and p.Arg229Gln caused a more prominent perinuclear localization of nephrin (fig. 3). This would likely lead to abnormal functioning of podocytes and/or the SD, since no adequate nephrin and/or podocin reaches the SD. Interestingly, our binding assay showed that the mutant p.Arg229Gln podocin binds more strongly on WT nephrin (fig. 5), in contrast to previous results [39]. The fact that we observed a similar behavior for variant p.Glu237Gln, in contrast to the WT podocin, is an indication for the validity of the results. We hypothesize that they exert an effect, which becomes evident only when co-inherited on the background of another glomerulopathy and only after many years during aging. Interestingly, Tory et al. [31], found evidence that p.Glu237Gln and p.Arg229Gln were 2 residues that were structurally connected. In the authors' words, 'Arg229 is stabilized in the core of the globular head domain by at least 2 hydrogen bonds involving Glu233, Glu237 or Asp244'. This reinforces our genetic and functional findings since the 2 SNPs studied here concern the same 2 residues. It does not escape our attention that the variant effects appear strong in the cellular setup tested (immunofluorescence studies and in vitro immunoprecipitation assays). This may relate to the cellular system used, including overexpression of the proteins under examination and may not reflect with absolute confidence the in vivo situation. Should the trafficking defect of nephrin be so conspicuous in the presence of the variants (fig. 3), and as suggested by the immunoprecipitation assay, it might lead to nephrotic syndrome in humans, which however is not the case. Notwithstanding these limitations, the combination of genetics and cellular studies in the presence of proper controls, provides convincing evidence and adds faith to the end result concerning the functional role of the variants presented here.

In conclusion, despite the relatively small patient cohort, and notwithstanding the need for independent confirmatory studies, our findings strengthen previously published results from our group and others, providing more evidence that certain non-synonymous variants in podocin are risk factors for proteinuria and renal impairment when found on the background of TBMN. At the same time, this co-inheriting of 2 genetic variants, 1 in the *COL4* and 1 in the *NPHS2* gene, could be viewed as reminiscent to digenic inheritance. The mechanism promoting this predisposition to kidney function impairment during aging might be related to the faulty cross-talk between the abnormal GBM and SD. This however, is highly speculative at the moment. These results conform to the rare variant-strong effect scenario for both p.Arg229Gln and p.Glu237Gln variants. These modifiers may be viewed as hypomorphic mutations, similar to what was demonstrated for hypomorphic mutations in the *PKD* genes that were found to be responsible for severe and autosomal dominant polycystic kidney disease that has an early onset [40]. In that paper, Bergmann et al. showed that some variants, which on their own are either recessive or totally neutral resulting in no perceptible phenotype, can lead to severe phenotype in childhood when co-inherited with mutations in the *PKD1/PKD2* genes, which normally are accompanied by late onset disease. Environmental factors such as smoking, high protein and sodium intake, in combination with the genetic background, may increase the risk for these patients even more. Gender difference also appeared as a significant contributing factor for disease progression between 'severe' and 'mild' patients in our cohort (table 1). This suggests that some gender-related behaviors (e.g. nutrition, smoking), sex hormones, Y chromosome sequences, etc., likely act as encumbering factors. Other existing kidney anomalies such as VUR may accelerate the progression, behaving in an additive manner together with genetic and environmental factors. In this study, we found evidence for the latter in one of the families studied (fig. 2). Our results showed that close clinical supervision and re-sequencing of the entire coding region of *NPHS2* gene in young TBMN patients, may alert for more careful long-term follow-up, for a closer genetic counselling and advice regarding life style and for more timely therapeutic interventions, all aiming to delay or prevent progression to ESRD. This work, also suggested that other rare podocin variants may exist, while it indicated that additional genetic factors definitely play a negative role of unknown degree in predisposing TBMN patients to an adverse disease outcome. This is simply based on the fact that many 'severe' patients did

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not carry either of the 2 variants. Equally, the putative positive role of other unknown genetic variants that protect mildly affected patients cannot be excluded.

Materials and Methods

Patients

The cohort details are presented in table 1. Patients with TBMN (122 patients, from 28 families) carrying a heterozygous *COL4A3* or *COL4A4* mutation, all of Greek-Cypriot origin, were categorized as 'mild' or 'severe'. No alternating thinning and thickening, lamellation, and splitting of the GBM were noted, which are pathognomonic features of classical AS, in 24 available biopsies. Also, hearing loss was either totally absent or a very rare occurrence at advanced ages, while ocular defects were never met. Out of the 122 described here, 102 patients overlapped with a previous study regarding variant *NPHS2*-p.Arg229Gln and familial hematuria [18]. This cohort was unique because it included a large number of patients with common mutations due to 2 major founder effects. Among 122 TBMN patients, 87 carried the *COL4A3*-p.Gly1334Glu mutation (from 17 families), 18 carried the *COL4A3*-p.Gly871Cys mutation (from 5 families), 7 carried the *COL4A4* c.(3854delG) mutation (from 2 families), 4 carried the *COL4A3*-p.Gly1077Asp mutation (from 1 family), 3 carried the *COL4A4*-p.Gly143Val mutation (from 1 family), 2 carried the *COL4A4*-p.Gly208Asp mutation (from 1 family) and lastly 1 carried the *COL4A3*-p.Gly484Arg mutation (from 1 family). Since young individuals with apparently mild disease could develop severe disease when growing older, we excluded patients with age <50 years without evidence of severe disease (table 1). TBMN patients with mild disease featured microscopic hematuria (MH) only or MH and clinically not significant proteinuria, with no CRF. TBMN patients with severe disease featured hematuria plus proteinuria ≥300 mg/24 h, with or without CRF/ESRD. CRF was defined as an elevated serum creatinine over 1.5 mg/dl. Severe patients with another concomitant renal disease or with a history of diabetes mellitus for at least 5 years, or at the extreme of body weight (outside ±2 SD of the cohort mean), were excluded. Ninety-six DNA samples from healthy individuals of the general population were used to find the frequency of the p.Glu237Gln allele. The p.Arg229Gln allele frequency in the general population was assessed as part of a previous work of our team [18].

The study was approved by the Cyprus National Bioethics Committee and participants gave their signed informed consent, unless they were included anonymously after testing for purely diagnostic purposes.

NPHS2 Re-Sequencing and Analysis of the Found Variants

The DNAs of 35 TBMN severe patients were directly re-sequenced for the coding region of the *NPHS2* gene. All 8 exons of the *NPHS2* gene were amplified by PCR using flanking intronic primers (available upon request) and subsequently re-sequenced. Direct re-sequencing was carried out using the Big Dye-terminator technology and the ABI-3130*xl* Genetic Analyzer. Sequences were evaluated with the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Calif., USA).

Identified non-synonymous variants were genotyped in additional 87 TBMN patients by DNA re-sequencing. Genotypic statistical analysis was performed by SPSS v.13 (IBM, New York, N.Y., USA). The significance level alpha was set to 0.05. Segregation analysis was performed for 2 variants in 2 families, by observing concordance and calculating the Mendelian probabilities.

The transcript reference codes for the genes relating to this work are *COL4A3* ENST00000396578, *COL4A4* ENST00000396625, *COL4A5* ENST00000361603, and *NPHS2* ENST00000367615.

The protein reference codes for the proteins relating to this work are *COL4A3* ENSP00000379823, *COL4A4* ENSP00000379866, *COL4A5* ENSP00000354505, and *NPHS2* ENSP00000356587.

Plasmid Vectors

A pCMV6-entry-Myc/DDK vector containing the full-length human podocin *(NPHS2)* cDNA was purchased from Origene (Maryland, USA). Additionally, cDNA of *NPHS2*-wt was subloned from pCMV6-*NPHS2*-wt-Myc/DDK to pCMV6-AC-HA vector. The pcDNA6/HIS containing the full-length human nephrin *(NPHS1)* cDNA was provided by Prof. T. Benzing, Cologne, Germany. The vectors were confirmed for the expression of C-terminal fusion of the podocin with the FLAG or HA tag and of N-terminal fusion of the nephrin with the V5 tag via western blotting.

Podocin missense mutations were introduced in the human podocin full-length cDNA using the QuikChange Site-Directed Mutagenesis Kit and according to the manufacturer's protocol (Stratagene, Calif., USA). Mutagenic primers were as follows:

NPHS2-p.Arg229Gln
For: CCACTATGAAGCGTCTCCTAGCACATCAATCCCTCACTGAAATTCTTCTAGAGAG;
Rev: CTCTCTAGAAGAATTTCAGTGAGGGATTGATGTGCTAGGAGACGCTTCATAGTG;
NPHS2-p.Glu237Gln
For: CTCACTGAAATTCTTCTACAGAGGAAGAGCATCGCCCAAGATG;
Rev: CATCTTGGGCGATGCTCTTCCTCTGTAGAAGAATTTCAGTGAG.

All constructs were re-sequenced to ensure that no undesired mutations had occurred during the mutagenesis procedure.

Cell Lines and Transfections

The human embryonic kidney cell line HEK293T was grown in Dulbecco's modified Eagle's medium (D-MEM) (Life Technologies, Calif., USA) supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin, in 37°C incubator with 5% CO_2.

The AB8/13 undifferentiated human podocyte cells (provided by Dr. M. Saleem, Bristol, UK) were incubated at 33°C at 5% CO_2 and cultured in RPMI medium, supplemented with 10% FBS (Invitrogen, Calif., USA), 1% of 100 U/ml penicillin/streptomycin (Invitrogen, Calif., USA), and 1% insulin-transferrin-selenium (Invitrogen, Calif., USA).

At 70% confluency, cells were transiently transfected with the vectors containing the podocin and nephrin cDNAs, WT or mutants, using Lipofectamine 2000 (Invitrogen, Calif., USA) and according to the manufacturer's instructions. After a 48-hour incubation period, cells were washed and subsequently lysed in Laemmli Sample Buffer. Transfection efficiency was assessed by immunofluorescence using anti-tag antibody (anti-FLAG and anti-HA, Santa Cruz, Calif., USA; an anti-V5 antibody, Invitrogen, Calif., USA) in a transfection control sample; transfection efficien-

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cy was typically about 60%. Podocin and nephrin expression was similar in all transfected cells as assessed by western blot, at each single experiment, using an anti-FLAG, an anti-HA or anti-V5 antibody respectively against the engineered terminal tags. Expression levels of both proteins after transfection strongly increased (30-fold) compared with non-transfected cells (data not shown).

Immunofluorescence

In order to determine the intracellular localization of the proteins of interest, AB8/13 undifferentiated human podocyte cells were cultured on glass coverslips and transiently co-transfected with nephrin and podocin cDNAs (WT or mutant), and further cultured for 48 h. Cells were washed and fixed in 3% paraformaldehyde in PBS for 10 min. After several washes in PBS, cells were permeabilized for 30 s in 0.1% Triton X-100, washed in PBS and blocked with PBS containing 1% Bovine Serum Albumin (BSA) for an hour at room temperature. Cells were incubated with the appropriate primary antibody, in blocking buffer, overnight at 4°C. After several washes, cells were incubated with the appropriate secondary antibody for an hour at room temperature. Then, after additional washes, coverslips were mounted on slides using Fluorescence Mounting Medium (DAKO, Glostrup, Denmark). Immunofluorescence preparations were analyzed on a Zeiss Axiovert 200M (Carl Zeiss, Jena, Germany) inverted fluorescence microscope equipped with Zeiss Axiovision 4.2 software and on a Zeiss LSM 710 laser scanning confocal microscope with Zen 2010 software. Digital images were recorded and composed using Adobe Photoshop 5.0 (Adobe Systems, Calif., USA).

Co-Immunoprecipitation

To determine the protein binding of the missense podocin mutants to WT podocin or nephrin, HEK293T cells were transiently co-transfected with the cDNAs of WT podocin or nephrin carrying the HA or V5 tag respectively, and WT or mutant podocin FLAG tagged and lysed after 48 h on ice in lysis buffer (50 mM Tris-HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100).

Lysates were centrifuged for 10 min at 13,200 *g* and the supernatants were then transferred in a new tube and incubated with ANTI-FLAG M2 (Sigma-Aldrich, St. Louis, Mo., USA) affinity gel for 3 h at 4°C.

The resin was then centrifuged and washed for several times with wash buffer (0.5 M Tris-HCl, pH 7.4, with 1.5 M NaCl). The immunoprecipitated FLAG fused podocin was eluted with 2× sample buffer (125 mM Tris-HCl, pH 6.8, with 4% SDS, 20% (v/v) glycerol, and 0.004% bromphenol blue). In order to minimize the denaturation of the antibody, no reducing agent was included in the sample buffer. The eluates were then applied for immunoblotting of nephrin and podocin.

Immunoblotting and Densitometry

Samples of HEK293 cells transiently transfected with WT or missense mutated podocin constructs, and the immunoprecipitated samples mentioned earlier were separated on 7.5% (SDS) gel electrophoresis under reducing conditions. They were then transferred overnight at 4°C to Immobilon polyvinylidene difluoride membranes (Millipore, Mass., USA) and incubated for 1 hour at room temperature with anti-FLAG (1:6,000), anti-HA (1:10,000) or anti-V5 (1:6,000) antibody. The membranes were then incubated for 60 min at room temperature with a 1:6,000 diluted HRP-labeled goat anti-rabbit or goat anti-mouse antibody. Proteins were detected using the Enhanced ChemiLuminescencePlus Blotting Detection system (GE Healthcare, Little Chalfont, UK) and were visualized using the ChemiDocXRS+ system (Biorad, Calif., USA). Band density was defined by the ImageJ Software (NIH, Md., USA). Statistical analysis was performed with the GraphPad Prism v.5 (GraphPad software, Inc., La Jolla, Calif., USA).

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Disclosure Statement

None to declare.

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