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The mutation-dependent pathogenicity of *NPHS2*, R229Q: a guide for clinical assessment

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Abstract

NPHS2, encoding podocin, is the major gene implicated in podocytopathies. Its c.686G>A, R229Q variant is the first human variant with a mutation-dependent pathogenicity: it is only pathogenic when trans-associated to specific mutations. Secondary to its high allele frequency in the European, South Asian, African and Latino populations, its benign trans-associations can be accidentally identified in affected patients. Distinguishing pathogenic and benign R229Q-associations is important for clinical management and genetic counseling, but can be challenging. In this paper, we present the currently known pathogenic and benign associations, and show that a rare R229Q-association can be considered pathogenic if the variant in trans meets the following criteria: it affects the 270-351 residues and alters but does not disrupt the oligomerization, its R229Q-association is found in a family with slowly progressing focal segmental glomerulosclerosis, but is expected to be rare in the general population ($<1:10^6$). We show that >15% of the R229Q associations identified so far in patients are benign.

Main text

Variants in autosomal recessive disorders cause typically partial or complete loss-of-function. Their pathogenicity therefore does not depend on the trans-associated mutation. The *NPHS2*, c.686G>A, p.R229Q is the first human variant discovered with a mutation-dependent pathogenicity: it is only pathogenic when trans-associated to specific mutations ([R229Q];$[mut]_{pat}$) (Tory et al., 2014). These mutations exert a dominant negative effect on R229Q podocin by blocking its membrane trafficking through an altered heterooligomerization (Straner et al., 2018; Tory et al., 2014). As a low amount of R229Q homooligomers are still expected to be formed and reach the cell membrane, the pathogenic associations cause the least severe *NPHS2*-related phenotype: slowly progressing focal segmental glomerulosclerosis (FSGS), typically diagnosed in the second or third decade of life and leading to end-stage renal disease between 10 and 50 years of age (Berdeli et al., 2007; Karle et al., 2002; Lipska et al., 2013; Machuca et al., 2009; Ruf et al., 2004; Sadowski et al., 2015; Santin et al., 2011; Tsukaguchi et al., 2002). Since no siblings with [R229Q];$[mut]_{pat}$ were ever found with discordant phenotype (Berdeli et al., 2007; Buscher et al., 2010; Hinkes et al., 2007; Ismaili et al., 2009; Karle et al., 2002; Machuca et al., 2009; Ruf et al., 2004; Sadowski et al., 2015; Santin et al., 2011; Tonna et al., 2008), we consider R229Q to be completely penetrant in these specific R229Q-associations. However, it is neither pathogenic in the homozygous state (Kerti et al., 2013), nor when trans-associated to the majority of the mutations ([R229Q];$[mut]_{ben)}$ (Tory et al., 2014).

Distinction of pathogenic and benign associations is difficult in clinical practice for two reasons. Firstly, the phenotype of *NPHS2*-related steroid-resistant nephrotic syndrome (SRNS) is not specific for *NPHS2*. The etiology of SRNS/FSGS is highly heterogeneous: a large proportion is immune-mediated and more than 30 causal genes have been identified in the hereditary podocytopathies. Therefore, *NPHS2* mutations are responsible for only 6.7%

(120/1783) in a worldwide cohort of patients diagnosed with SRNS/FSGS before 25 years of age, and R229Q associations are causal in only 1.18% (21/1783) (Sadowski et al., 2015). Hence, approximately 100 patients with SRNS/FSGS have to be screened to identify one with a causal R229Q-association. Secondly, the R229Q variant is common in the European, South Asian, African and Latino populations (Table 1), allowing the accidental identification of compound heterozygous patients with benign R229Q-associations. The low rate of the pathogenic R229Q-associations in SRNS/FSGS and the relatively high risk of the accidental identification of a non-pathogenic R229Q association explain, as we show here, that a relatively large proportion of the R229Q associations (18/106 [17%]) identified in patients are in fact benign.

Patients with [R229Q];$[mut]_{ben}$ suffer from other forms of SRNS/FSGS, which may respond to immunosuppressive treatment and recur after transplantation. Considering a benign R229Q association to be pathogenic is therefore particularly harmful, as it may falsely lead to the diagnosis of monogenic podocytopathy and thus to the withdrawal of immunosuppressive treatment in an immune-mediated form of the disease. Furthermore, given the large number of families with *NPHS2* mutations (>500 published), and the high frequency of the R229Q carriers in several populations, distinction of pathogenic and benign R229Q-associations is also crucial for genetic counseling.

Here we summarize our current knowledge about the pathogenic R229Q associations to help their distinction in clinical practice. We use different approaches: genetic studies in families with affected children, population-genetic analysis and biophysical analysis of the heterooligomers (Straner et al., 2018; Tory et al., 2014).

Table 1. Allele frequency (AF) of R229Q and pathogenic *NPHS2* mutations, and expected frequency of patients with [R229Q];[mut] in different populations (gnomAD) (Lek et al., 2016).

	European	East Asian	South Asian	African	Latino
AF of R229Q	3.6%	0.01%	2.7%	0.54%	1.3%
cumulative AF of all other pathogenic *NPHS2* mutations	0.224%	0.081%	0.077%	0.142%	0.178%
expected frequency of pts with [R229Q];[mut]	1/6 000	1/6 M	1/24 000	1/65 000	1/21 000
cumulative AF of mutations pathogenic w R229Q	0.004%	0.032%	0.018%	0.007%	0
expected frequency of pts with $[R229Q];[mut]_{pat}$	1/350 000	1/15 M	1/100 000	1/1.3 M	NA
ratio of pathogenic/all [R229Q];[mut] associations	0.017	0.4	0.24	0.05	NA

Genetic studies in affected families

The high allele frequency of R229Q in Europe allowed us to exclude the pathogenicity of several R229Q associations by screening unaffected mutation carrier parents and uncles of children with *NPHS2* mutations for R229Q. We thus found the R138*, R138Q, R168H, c.534+1G>T, R238S and V290M mutations to be trans-associated to R229Q in unaffected adults (Table 2) (Tory et al., 2014).

Population-genetic approach

As introduced above, secondary to the high allele frequency of R229Q, benign R229Q-associations can be accidentally identified in affected individuals. However, only the dominant negative mutations are causal and therefore enriched among patients with [R229Q];[mut] (Tory et al., 2014). The dominant negative effect of a mutation can be thus proved based on its significant enrichment among patients with [R229Q];[mut] as compared to patients with biallelic mutations ([mut];[mut]) (Table 2). Given that only a few mutations

are pathogenic with R229Q, and these are rare among the *NPHS2* mutations, a significant enrichment can be easily achieved by a relatively small number of patients with [R229Q];[mut] (currently n≥2/106, Table 2). This approach allowed us to prove the pathogenicity of A284V, A288T, R291W, A297V, E310K, E310V, L327F, Q328R and F344Lfs*4 (Straner et al., 2018; Tory et al., 2014).

The population-genetic approach can also serve to support the benign nature of an R229Q-association. A mutation that is benign with R229Q is only accidentally identified among patients with [R229Q];[mut]. Therefore, these associations are underrepresented in the patient population. This approach is informative only for the frequent variants: the allele count of a variant has to currently exceed n=32/878 alleles among the patients with [mut];[mut] to potentially achieve a significant underrepresentation ($p<0.05$) among the 106 patients with [R229Q];[mut]. Only the R138Q, L156Ffs*11 and R168H mutations are currently frequent enough to prove the benign nature of their R229Q-association by this method (Table 2).

Table 2. R229Q-associations with a known benign or pathogenic nature based on family studies or a population-genetic approach.

mutation trans-associated to R229Q		identified unaffected individuals /families	AC in pts w [R229Q];[mut]	AC in pts w [mut];[mut]	enrichment in pts w [R229Q];[mut]	F_{max} of pts w [RQ];[mut] (region)	age at dg/ ESRD (years)	pathogenicity w R229Q
nucleotide	**consequence**		**∑= 106 alls of 106 pts**	**∑=878 alls of 439 pts**	**$[AF_{[R229Q];[mut]})/(AF_{[mut];[mut]})]$ ($p^{\#}$)**			
c.413G>A	R138Q	**1/1**	6	281 (182pt)	**0.18x ($3.9x10^{-7}$)**	1/12k (Europe)	3.8-34/34-40	benign
c.412C>T	R138*	**2/1**	0	24 (15pt)	0x (0.16)	1/780k (Europe)	NA	benign
c.467dupT	L156Ffs*11		0	59 (41pt)	**0x (0.002)**	1/65k (Europe)	NA	benign
c.503G>A	R168H	**1/1**	0	33 (21pt)	**0x (0.04)**	1/650k (Europe)	NA	benign
c.534+1G>T	abnormal transcript	**1/1**	0	4 (3pt)	0x (1)	0	NA	benign
c.714G>T	R238S	**1/1**	1	6 (4pt)	1.4x (0.5)	1/570k (South Asia)	ND	benign
c.851C>T	A284V	0	34	10 (5pt)	**28x ($6.6x10^{-23}$)**	1/1.5M (Europe)	0-39/8-50	pathogenic
c.855_856delAA	R286Tfs*17	**1/1 (SSNS)**	3	23 (17pt)	1.1x (0.75)	1/97k (Europe)	20-27/30-35	benign
c.862G>A	A288T	0	5	0	**NA - enriched ($1.6x10^{-5}$)**	1/1.5M (Europe)	12-28/9-40	pathogenic
c.868G>A	V290M	**2/2**	2	22 (18pt)	0.75x (1)	1/55k (Europe)	1.5-2.3/ND	benign
c.871C>T	R291W	0	5	3 (3pt)	**14x (0.0007)**	1/15M (East Asia)	0-3.5/10-34	pathogenic
c.890C>T	A297V	0	6	0	**NA - enriched ($2x10^{-5}$)**	1/1.4M (Africa)	4.3-34/3.6-31	pathogenic
c.928G>A	E310K	0	7	0	**NA - enriched ($2x10^{-7}$)**	0	0.6-30/3.2	pathogenic
c.929A>C	E310A	0	2	0	**NA - enriched (0.01)**	0	4.1-18/34	pathogenic
c.929A>T	E310V	0	4	0	**NA - enriched (0.0001)**	0	0.2-5/8.5-11.9	pathogenic
c.979C>T	L327F	0	4	0	**NA - enriched (0.0001)**	1/780k (Europe)	3-25/29	pathogenic
c.983A>G	Q328R	0	4	0	**NA - enriched (0.0001)**	0	6-10/ND	pathogenic
c.1032delT	F344Lfs*4	0	6	0	**NA - enriched ($2X10^{-5}$)**	0	0.6-17.6//ND	pathogenic

AC: allele count, AF: allele frequency, alls: alleles, ESRD: end-stage renal disease, F_{max}: highest expected frequency, hom: homozygous, k: thousand, M: million, NA: not applicable, ND: no data, NS: not significant, pt(s): patient(s), RQ: R229Q, SSNS: steroid-sensitive nephrotic syndrome, w: with, $^{\#}$significance of Fisher's exact test. Patients' data are compiled from the medical literature (Al-Hamed et al., 2013; Berdeli et al., 2007; Bertelli et al., 2003; Buscher et al., 2010; Caridi et al., 2001; Chernin et al., 2010; Jungraithmayr et al., 2011; Karle et al., 2002; Lipska et al., 2013; Machuca et al., 2009; Megremis et al., 2009; Ruf et al., 2004; Sadowski et al., 2015; Santin et al., 2009; Santin et al., 2011; Tonna et al., 2008; Tsukaguchi et al., 2002; Voskarides et al., 2008).

The dominant negative effect of a mutation can also be excluded based on its high allele frequency in the general population. Even the well-known, relatively frequent dominant negative mutations are extremely rare (Table 1): the expected frequency of their R229Q-association does not exceed $1:10^6$ in any population (Table 2). Though studies on 54 European patients have been published with the well-known pathogenic R229Q-associations (Table 2), the cumulative allele frequency of these mutations is only 4/110 000 in Europe. Therefore, variants with a relatively high allele frequency in the general European population are expected to be benign with R229Q. For this reason, the dominant negative effect of the frequent 3' variants: I263L, E264Q, G273W, R291Q, V319L, Q320P, K377R or that of the known 3' pathogenic mutations: R286Tfs*17, V290M, A317Lfs*31 is highly unlikely (Table 3).

Table 3. The underrepresentation of 3' variants among European patients with [R229Q];[mut] as compared to the general European population supports the benign nature of their R229Q-association. Mutations that are pathogenic in the homozygous state are in bold.

		allele count		**p[#]**
		European pts w [R229Q];[mut]	European general population (/110 000 alleles – gnomAD)	vs. DN mutations
Dominant negative mutations		54	4	
Frequent 3' variants with no DN effect	I263L	0	7	$4.7x10^{-7}$
	E264Q	0	31	$6.1x10^{-20}$
	G273W	0	3	0.001
	R286Tfs*17	3	18	$2.9x10^{-11}$
	V290M	2	32	$5.1x10^{-18}$
	R291Q	0	10	$3.4x10^{-9}$
	A317Lfs*31	0	3	0.001
	V319L	0	3	0.001
	Q320P	0	3	0.001
	K377R	0	22	$5.5x10^{-16}$

DN: dominant negative, pts: patients, w: with, [#]significance of Fisher's exact test
Patients' data are compiled from the medical literature (Machuca et al., 2009; Sadowski et al., 2015; Tonna et al., 2008)

Biophysical approach

Analysis of the wild type and pathogenic R229Q-associations allowed us to understand the molecular and cellular basis of the pathogenic interactions. According to our structural model, which is primarily based on the crystal structure of the partially homologous stomatin (Tory et al., 2014), the residues 161-332 of podocin form a globular PHB domain (161-282) and a mainly helical tail region (C-terminal tail: CTT) comprising two helical segments (H1: 282-313, H2: 317-330). Secondary structure prediction indicates that the final stretch of the C-terminal contains a further helix (H3: 344-350) and an unstructured loop (351-383) at the terminus (Straner et al., 2018). The R229 is located on the PHB domain, in a position facing the CTT (Fig. 1). The R229Q change results in the formation of new hydrogen bonds between the PHB and CTT domains, resulting in less flexible monomers (Tory et al., 2014). Since the CTT contains a number of hydrophobic amino acids in a distribution reminiscent of the interaction motifs of a coiled-coil association (a superhelix of helices), we proposed that the dimers are formed by the antiparallel inter-twine of the H1 helices, reinforced by the wrapping H2 helices (Fig. 1) (Tory et al., 2014). In a recent study, we could gain experimental support for this notion, showing that the H1 segment in itself forms a coiled coil, oligomers are assembled from dimeric pairs, and that while dimerization is driven by the H1 and H2 helices, oligomerization is mediated by the short connecting segment between H2 and H3 and H3 itself (Straner et al., 2018). Using a model based on these findings, we demonstrated that the altered pliability of R229Q podocin affects the evolving dimer structures, but this effect is only significant if the associated mutation falls on the winding interaction surface of the coiled-coiled CTTs (as in positions 284, 288, 295, 297 and 327, but not the outward facing 290) (Fig.1A, B). We also found an overall increased FRET efficiency in the R229Q heterooligomers as compared to the wild type heterooligomers suggesting formation of more compact, possibly more tightly bound R229Q oligomers (Straner et al.,

2018). Thus, the dominant negative effect seems to result on one hand from the altered conformation and on the other hand, from the increased binding affinity of the pair or oligomer. The A284V, A288T, R291W, A297V, L327F variants are examples of such changes where the increase in size and hydrophobicity (or H-bonding potential) at a buried site may explain both consequences. Further means of altering the oligomerization might be the introduction of helix breaker amino acids like Pro in the middle of helical stretches (A300P, L305P) or changing the charge pattern of the outward facing side of the coiled-coil (R306W, E310K, E310A, E310V) (Table 2 and Fig.1C).

Hence, to be pathogenic with R229Q, a mutation has to form strong association with R229Q in a manner that alters the conformation of the oligomer. Therefore, missense mutations N-terminal to L270 (the stem of H1 and residues interacting with it already starts within the PHB domain) or C-terminal to S351 (end of H3) are not expected to influence the oligomerization and to be pathogenic with R229Q.

Not all substitutions are pathogenic within the L270-S351 region either (Fig. 2): V290M despite being located in the first oligomerization site, between A288T and R291W which are both pathogenic with R229Q, exerts no dominant negative effect, as shown by several aspects (Tables 2, 3). This is in accordance with its position in the coiled coil as it points toward the solvent and away from the dimerization surface leaving the latter untouched (Fig. 1) (Tory et al., 2014). Similarly, the benign nature of G273V and V319L variants (Table 3, Fig. 2) can be explained by their pointing away from the dimerization/oligomerization interaction surfaces.

Mutations that result in changes on the interaction surface can also be benign if the switch does not cause substantial change in size or interacting capacity, such as R291Q (Table 3), affecting the same amino acid as the dominant negative R291W (Table 2, Fig. 2). Since the Gln sidechain in this position can support both crucial H-bonds that the wild type Arg forms (Fig. 1), no significant change in the dimer structure is expected.

The third group of mutations which may not exert a dominant negative effect, despite appearing within the L270-S351 region, are the truncating mutations which disrupt the oligomerization. As most of the C-terminal is encoded by the last exon, a premature stop within this region (downstream to codon 274) is not expected to induce mRNA decay. The R286Tfs*17 or A317Lfs*31 mutations disrupt the sequence of either H1 (R286Tfs*17) or H2 (A317Lfs*31) causing the loss of either both or only the second oligomerization site (Fig. 2). Accordingly, the R286Tfs*17 podocin forms only monomers while the A317Lfs*31 podocin is expected to form only dimers (Straner et al., 2018). This can explain why they cannot (efficiently) block the membrane targeting of R229Q podocin (Straner et al., 2018) (Tables 2, 3). On the other hand, the F344Lfs*4 (Table 2) and the L346Yfs*2 mutations (Table 4), which affect but do not abolish the second oligomerization site can exert a dominant negative effect on R229Q (Fig. 2) (Straner et al., 2018).

Figure 1. Domains and residues implicated in the podocin dimerization.

A. PHB, H1 and H2 domains of the calculated podocin dimer model in frontal and lateral view. Sidechain atoms of R229Q in the PHB domain and all residues of the dimerization region (H1and H2 helix) are shown without their nonpolar hydrogens.

R229 and amino acids discussed in the text are shown in space-filling representation (CPK). B. The hydrophobic interior created around Ala284 and Ala288 of H1 by the surrounding Val280, Leu305, Ala308, Leu312, Leu321, Leu324 of the two interacting chains. Glu310 at the end of H1 interacts with the neighboring Arg306, as part of a charged patch also containing Glu303. C. Arg291 wedged between Gln328 and Glu298 of the facing monomer creating a double cross-link at the middle of the dimerization site. Inserting a hydrophobic and very bulky Trp at this position (as in case of the pathogenic R291W mutation) would significantly rearrange this organizational center, however a Gln (as in benign variant R291Q) might be able to perform similar bridging function as the wild type Arg.

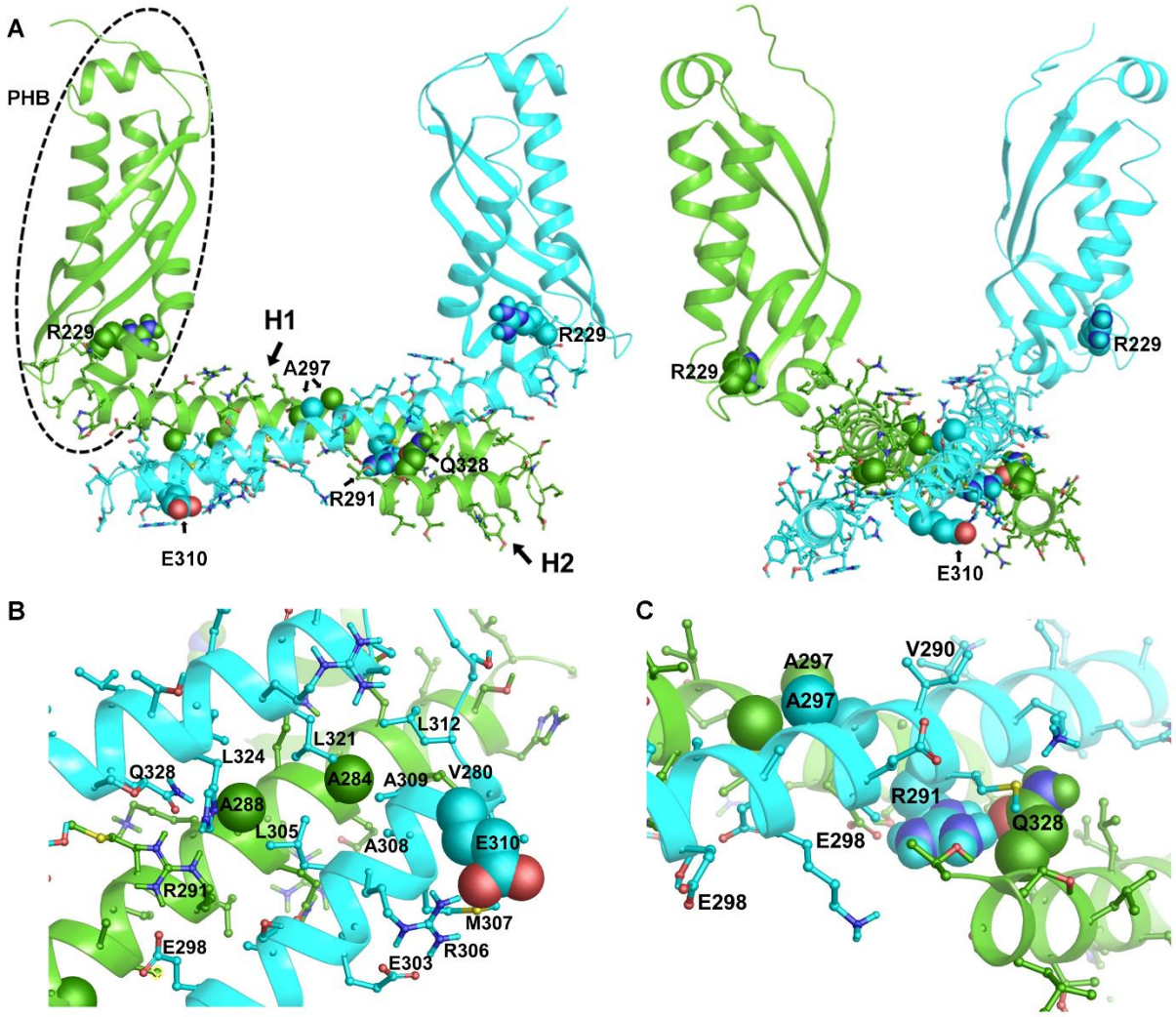

A
PHB
R229
H1
A297
R229
Q328
R291
E310
H2
R229
R229
E310
B
L324
L321
L312
Q328
A284
A309
V280
A288
L305
A308
E310
R291
M307
E298
E303
R306
C
A297
A297
V290
R291
Q328
E298
E298

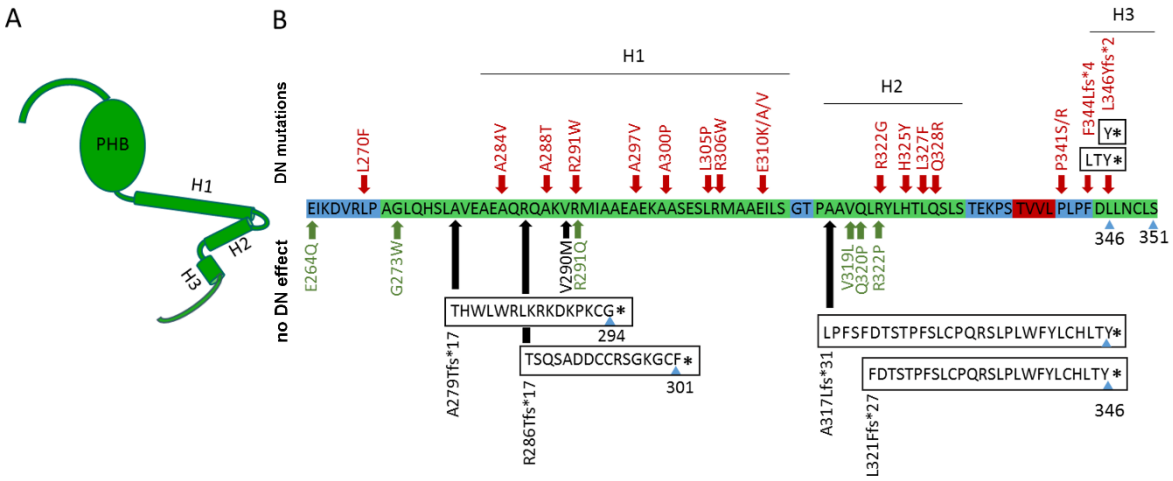
Table 4. Pathogenicity of rare R229Q associations

mutation trans-associated to R229Q		ethnic origin of pt	AC in pts [RQ];[mut]	AC in pts [mut];[mut]	enrichment in pts w [RQ];[mut]	F_{max} of pts w [RQ];[mut] (region)	age at dg/ ESRD (years)	predicted pathogenicity w R229Q
nucleotide	consequence		∑= 106 alls of 106 pts	∑=878 alls of 439 pts	$(AF_{[RQ];[mut]})/(AF_{[mut];[mut]})$] ($p^{\#}$)			
c.376_377insT	K126Ifs*7	Turkish	1	0	NA - enriched (0.1)	1/570k (South Asia)	ND	benign
c.451+3A>T	altered transcript	German	1	1	8.3 (0.2)	1/630k (Latin)	14.8/ND	benign
c.643C>T	Q215*	French	1	7	1.2 (0.6)	1/590k (Europe) 1/570k (South Asia)	0.8/43.5	benign
c.810G>T	L270F	European-American	1	0	NA - enriched (0.1)	0	38/ND	pathogenic
c.826_833dup CACTCACT	A279Tfs*17	European	1	0	NA - enriched (0.1)	0	8/ND	benign
c.841G>A	E281K	European	1	0	NA - enriched (0.1)	0	siblings: I. 3/ND II. 22.4/ND	pathogenic
c.883G>A	A295T	Greek	1	2 (1pt)	4.1x (0.3)	0	4/ND	pathogenic
c.898G>C	A300P	European	1	0	NA - enriched (0.1)	0	siblings: I. 60/ND II. 16.2/ND	pathogenic
c.914T>C	L305P	Greek	1	0	NA - enriched (0.1)	0	ND	pathogenic
c.916A>T	R306W	European	1	0	NA - enriched (0.1)	0	siblings I. 14.5/ND II. ND/ND	pathogenic
c.961delC	L321Ffs*27	Slovenian	1	0	NA - enriched (0.1)	0	13.3/ND	benign (?)
c.964C>G	R322G	South American	1	2 (1pt)	4.1x (0.3)	1/210k (Europe)†	34/38	pathogenic
c.965G>C	R322P	Indian	1	0	NA - enriched (0.1)	1/140k (South Asia)	1.6/ND	benign (?)
c.973C>T	H325Y	Italian	1	0	NA - enriched (0.1)	0	18.8/21.2	pathogenic (?)
c.1021C>T	P341S	Hungarian	1	0	NA - enriched (0.1)	0	8/no ESRD (12)	pathogenic
c.1022C>G	P341R	Finnish	1	0	NA - enriched (0.1)	0	6/19	pathogenic
c.1036delC	L346Yfs*2	Australian	1	0	NA - enriched (0.1)	0	three siblings 15-28/29-32	pathogenic

alls: alleles, F_{max}: highest expected frequency, NA: not applicable, ND: no data, pt(s): patient(s), RQ: R229Q, w: with, $^{\#}$significance of Fisher's exact test. † The AF of R322G cannot be properly estimated as only 15k alleles are available in gnomAD. Patients' data are compiled from the medical literature (Berdeli et al., 2007; Lipska et al., 2013; Machuca et al., 2009; Megremis et al., 2009; Sadowski et al., 2015; Suvanto et al., 2017; Tonna et al., 2008; Tsukaguchi et al., 2002; Voskarides et al., 2008)

Figure 2. Localization of C-terminal variants with and without dominant negative effect on R229Q podocin.

A) Schema of the globular PHB domain and the three C-terminal helical regions of podocin. B) Wild type residues 264-351 of podocin, highlighted according to their loop- (blue), strand- (red) or helix-forming (green) propensity (Straner et al., 2018). The variants with a dominant negative effect are shown in red above the podocin sequence. Variants that are pathogenic in the homozygous state but not in-trans with R229Q are shown below the sequence, in black with the frameshift sequences framed. Benign variants, which are not pathogenic neither with R229Q, nor in the homozygous state are in green.



Pathogenicity of the rare R229Q-associations

While in populations with low R229Q frequency the accidental identification of a benign R229Q association is of negligible risk, the pathogenicity of the rare R229Q-associations (Table 4) can be challenging to assess in populations with frequent R229Q (Table 1). Based on the presented observations, we propose to consider a mutation to be pathogenic with R229Q if it meets all of the following criteria:

1) It causes amino acid change in the region of oligomerization (residues 270-351, Fig. 2) that results in change of size, polarity or hydrogen bonding capacity.
2) It does not disrupt the oligomerization. Thus, the A279Tfs*17 mutation (Table 4, Fig. 2) disrupting both oligomerization sites is not expected to be pathogenic. We expect the [R229Q];[L321Ffs*27] to be probably also benign, as L321Ffs*27 disrupts the second oligomerization site similarly to A317Lfs*31 (Table 4, Fig. 2).
3) The expected frequency of individuals with [R229Q];[mut] in the general populations is lower than $1:10^6$. Otherwise, it would be a frequent and already known association (Tables 2, 4).
4) It is enriched (even non-significantly) among patients with [R229Q];[mut] as compared to patients with [mut];[mut], i.e. a mutation of a novel R229Q-association should not have been reported to be associated to another *NPHS2* mutation in more than a single case in populations with frequent R229Q.
5) The [R229Q];[mut] association segregates with the disease in the family.
6) The associated phenotype corresponds to the R229Q-associated nephropathy with no overt edema, FSGS on histology and low rate of progression (leading to ESRD between 10 and 50 yrs of age).
7) The mutation is in trans with R229Q. Particular attention has to be paid to P341R, which was reported in the heterozygous state in a patient with homozygous R229Q (Suvanto et al., 2016), indicating that this mutation appeared on an R229Q allele. A patient with heterozygous R229Q and P341R is therefore primarily expected to carry these variants in cis, which is particularly important to verify. We consider the *trans*-association of R229Q and P341R to be pathogenic because this association corresponds to the above criteria (Table 4).

Based on these criteria, we predicted the pathogenicity of the so-far published rare associations (Table 4). If the distinction is ambiguous (R322P, H325Y and L321Ffs*27), we propose to consider the association to be benign, usage of immunosuppression, and to consider the lack of complete remission as an argument for the pathogenicity of the R229Q-association.

In summary, out of the 106 patients with [R229Q];[mut] (Al-Hamed et al., 2013; Berdeli et al., 2007; Bertelli et al., 2003; Buscher et al., 2010; Caridi et al., 2001; Chernin et al., 2010; Jungraithmayr et al., 2011; Karle et al., 2002; Lipska et al., 2013; Machuca et al., 2009; Megremis et al., 2009; Ruf et al., 2004; Sadowski et al., 2015; Santin et al., 2009; Santin et al., 2011; Tonna et al., 2008; Tsukaguchi et al., 2002; Voskarides et al., 2008), we found 18 patients (17.9%) to carry benign mutations (Tables 2, 4). Most of the patients with [R229Q];$[mut]_{ben}$ (12/18) are European, in accordance with the highest allele frequency of R229Q and the highest rate of *NPHS2* mutations which are non-pathogenic with R229Q in this population (Table 1). The three most commonly identified benign R229Q-associations are formed by R138Q, R286Tfs*17 and V290M. The benign nature of their association (Tables 2, 3), explain the recurrence the nephrotic syndrome after transplantation in patients with [R138Q];[R229Q] (Tonna et al., 2008) and the steroid-responsiveness of the nephrotic syndrome in a patient with [R229Q];[R286Tfs*17] (He et al., 2007).

It is unlikely that R229Q was the only variant with a mutation-dependent pathogenicity, as we found several C-terminal podocin variants to be influenced by the coexpressed podocin variant (Straner et al., 2018). The high frequency of R229Q greatly facilitated its identification as a subject of interallelic interactions and the distinction of its pathogenic and benign associations. This will be more challenging in case of rare C-terminal variants. Interallelic interactions of 3' *NPHS2* mutations have to be considered in compound

heterozygous individuals when the phenotype does not match the *NPHS2*-related podocytopathy.

In conclusion, here we have summarized the pathogenicity of the common R229Q-associations. The pathogenicity of the rare associations has to be assessed by a combined population-genetic, molecular, segregation-analysis and clinical approach.

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