

# Specific Podocin Mutations Correlate with Age of Onset in Steroid-Resistant Nephrotic Syndrome

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## ABSTRACT

Mutations in the gene encoding podocin (*NPHS2*) cause autosomal recessive steroid-resistant nephrotic syndrome (SRNS). For addressing the possibility of a genotype–phenotype correlation between podocin mutations and age of onset, a worldwide cohort of 430 patients from 404 different families with SRNS were screened by direct sequencing. Recessive podocin mutations were present in 18.1% (73 of 404) of families with SRNS, and 69.9% of these mutations were nonsense, frameshift, or homozygous R138Q. Patients with these mutations manifested symptoms at a significantly earlier age (mean onset <1.75 yr) than any other patient group, with or without podocin mutations, in this study (mean onset >4.17 yr). All but one patient affected by truncating or homozygous R138Q mutations developed SRNS before 6 yr of age. Patient groups with other recessive podocin mutations, with single heterozygous podocin mutations, with sequence variants, and with no podocin changes could not be distinguished from each other on the basis of age of onset. In conclusion, nephrotic syndrome in children with truncating or homozygous R138Q mutations manifests predominantly before 6 yr of life, and the onset of disease is significantly earlier than for any other podocin mutations. Because the age of onset can vary by several years among those with identical mutations, additional factors may modify the phenotype.

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Nephrotic syndrome (NS) is a common childhood kidney disease caused by impaired glomerular function, which results in proteinuria, hypoalbuminemia, edema, and hyperlipidemia. Although underlying causes of NS are numerous, standard steroid treatment regimens are used to classify idiopathic forms of NS clinically. On the basis of the patients' response to steroid treatment, NS is descriptively classified as steroid-sensitive NS (SSNS) or steroid-resistant NS (SRNS).

Positional cloning of various genes as mutated in patients with SRNS in recent years has led to the surprising finding that up to 28% of sporadic cases of SRNS are caused by recessive mutations of *NPHS2*.<sup>1</sup> Mutations causing recessive NS in children were found in nephrin (*NPHS1*),<sup>2</sup> podocin (*NPHS2*),<sup>3</sup> laminin- $\beta$ -2 (*LAMB2*), and phospho-

lipase C- $\epsilon$  (*PLCE1*).<sup>4–6</sup> Dominant mutations of exons 8 and 9 of the Wilms' tumor 1 gene (*WT1*) also cause childhood NS.<sup>7</sup> In adult patients with SRNS, dominant disease causing mutations in *ACTN4* and *TRPC6* were found.<sup>8–10</sup> Conjointly, these findings have shown that podocyte dysfunction affecting the glomerular slit diaphragm is a key feature of SRNS.<sup>11</sup>

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Among these SRNS genes identified so far, mutations in *NPHS2* encoding podocin represent the most frequent cause.<sup>1,12</sup> Patients with *NPHS2* mutations show histology of FSGS in approximately 80% of cases and progress to ESRD. Extensive studies have documented that the detection of two *NPHS2* mutations is clinically relevant, because affected patients do not respond to standard steroid treatment.<sup>1</sup> Thus, adverse effects of such treatment attempts can be avoided in children with recessive *NPHS2* mutations. In addition, patients with *NPHS2* mutations have a reduced risk (8 versus 33%) for recurrence of FSGS in a kidney transplant.<sup>1,13</sup>

*NPHS2* mutations have been found in patients presenting at a wide range of ages, in contrast to *NPHS1* (nephrin) mutations, which lead to congenital NS with onset within the first 3 mo of life. The reason for this phenotypic variability of patients with *NPHS2* mutations remains elusive. In our recent study on NS manifesting in the first year of life (NSFL), we identified *NPHS2* mutations as the most frequent cause of both congenital (0 to 3 mo) and infantile (3 to 12 mo) NS among 89 European children with NSFL.<sup>14</sup> In 94.1% of patients with NSFL caused by *NPHS2* mutations, we found nonsense, frameshift, or homozygous R138Q mutations. These findings and the suggestive data by Weber *et al.*<sup>13</sup> led to the question of whether specific *NPHS2* mutations determine the age of initial onset of SRNS.

To evaluate this genotype–phenotype correlation among patients with *NPHS2* mutations, we examined 430 patients from 404 families from a worldwide cohort who presented with SRNS between 0.0 and 21.0 yr of age for mutations in *NPHS2* by direct sequencing of all eight exons. In this study, we demonstrate that nonsense, frameshift, or homozygous R138Q mutations are frequent recessive mutations of *NPHS2*. We show that these *NPHS2* mutations are found in patients

with a significantly earlier onset than in patients with other recessive mutations, single mutations, or sequence variants of *NPHS2*. We observe that identical mutations lead to onset of SRNS within a range of several years and speculate that additional factors modify the *NPHS2* phenotype. These findings will be important for the prognostic evaluation and management of renal replacement therapy in children with SRNS.

## RESULTS

### Frequency of *NPHS2* Mutations

A total of 430 patients with SRNS from 404 families were analyzed for mutations of *NPHS2* by direct DNA sequencing. Mutations in *NPHS2* were present in 18.1% (73 of 404) of all families and affected 82 patients (Table 1). In families with more than one affected member, mutations were present in 39.1% (nine of 23 families). In families with one affected child, mutations were present in 16.8% (64 of 381 families; data not shown). The R138Q mutation that has been described as a European founder mutation<sup>3</sup> was found in 57.5% (42 of 73) of families with the presence of two disease-causing mutations (Table 1, groups A through D; Supplementary Table 1). In 5.1% (21 of 404) of all families, only one single heterozygous mutation was detected (Table 1, groups E and F; Supplementary Table 1).

The distribution of the four groups with two recessive mutations (A through D) among 73 families with *NPHS2* mutations and clinical data were as follows (Table 1): A, 39.7% (29 of 73) with one truncating mutation (nonsense or frameshift) in combination with any other mutation; B, 30.1% (22 of 72) with a homozygous R138Q mutation; C, 12.3% (nine of 73) with one R138Q mutation in combination with one missense

**Table 1.** A total of 430 patients from 404 families with SRNS analyzed for mutations and sequence changes in *NPHS2*<sup>a</sup>

Group	<i>NPHS2</i> Mutation	Affected Individuals		Affected Families		Mean Onset (yr)	Onset Range (yr)
		Total	Onset known	Total	Onset known		
A	Truncating × any <sup>b</sup>	32	31	29	28	1.75 <sup>c</sup>	0.0 to 9.1
B	R138Q × R138Q	27	26	22	21	1.77 <sup>c</sup>	0.0 to 5.4
C	R138Q × missense	10	9	9	8	5.95	0.0 to 14.3
D	Missense × missense	13	12	13	12	4.17	0.0 to 16.6
		82	78	73	69	2.61	0.0 to 16.6
E	Single × R229Q	14	13	13	12	6.74	0.8 to 16.3
F	Single × ?	9	9	8	8	8.12	1.6 to 14.7
G	R229Q × R229Q	2	2	2	2	6.48	0.0 to 13.0
	R229Q × ?	24	23	24	23		
		49	47	47	45	6.87	0.0 to 16.3
H	No mutation	299	267	284	253	6.4	0.0 to 21.0
Total		430	392	404	367	5.71	0.0 to 21.0

<sup>a</sup>A total of 430 patients from 404 families with SRNS were analyzed by direct exon sequencing for sequence changes in *NPHS2*. Clinical data on 392 patients from 367 families allowed for correlation on *NPHS2* sequence variants with age of onset. Patients were categorized into patients with two disease-causing *NPHS2* mutations (A through D), patients with a single *NPHS2* mutations and/or sequence variant R229Q (E through G), and patients without *NPHS2* variants (H).

<sup>b</sup>Group A includes 14 individuals from 11 families with a single R138Q mutation accompanying the truncating mutation. The total number of families/individuals with R138Q mutations is 42/5.

<sup>c</sup>Age of onset for both groups A and B was significantly lower than for all other groups ( $P < 0.01$ ).

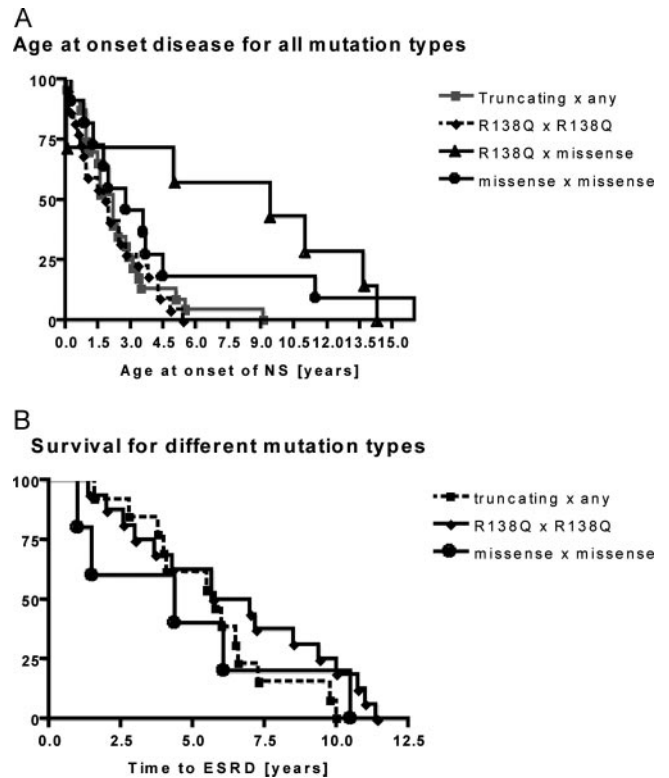
mutation; and D, 17.9% (13 of 73) with two missense mutations other than R138Q (Table 1). Six previously unpublished *NPHS2* mutations were found (Supplementary Table 1).

In 47 families, we detected only one single *NPHS2* mutation or the sequence variant R229Q of unknown significance (Table 1, groups E through G). In these 47 families, one heterozygous mutation in combination with the R229Q sequence variant of unknown significance was present in 27.7% (13 of 47; E). In 17.0% (eight of 47) of families, one heterozygous *NPHS2* mutation only was present (F), and in 55.3% (26 of 47) of families, the R229Q sequence variant only was present heterozygously (24 of 47) or homozygously (G; Table 1). The frequency of R229Q variants among all 404 families with SRNS in this study was 9.2% (37 of 404) in the heterozygous state and 0.5% (two of 404) in the homozygous state.

### Correlation of *NPHS2* Mutations and Age of Onset

Age at onset of SRNS was documented for 392 patients from 367 families and ranged from 0.0 to 21.0 yr. Two disease-causing *NPHS2* mutations were found in 19.9% (78 of 392) of these patients. The relative frequency with which any two disease-causing *NPHS2* mutations were detected declined with increasing age at manifestation.

We then evaluated genotype–phenotype correlations between type of mutation and age of onset (Table 1). This correlation showed significantly earlier onset of SRNS in children with two disease-causing *NPHS2* mutations (A through D; mean 2.61 yr; range 0.0 to 16.6 yr) than in children with single heterozygous *NPHS2* mutations and sequence variant R229Q (E through G; mean 6.87 yr; range 0.0 to 16.3 yr;  $P < 0.0001$ ). Children with two recessive *NPHS2* mutations (A through D) also manifested significantly earlier when compared with children without *NPHS2* sequence variants (H; mean 6.4 yr; range 0.0 to 21.0 yr;  $P < 0.0001$ ). In particular, the presence of two truncating mutations (nonsense or frameshift) in *NPHS2* (A) was associated with much earlier onset (mean 1.75 yr; range 0.0 to 9.1 yr). Likewise, the homozygous presence of the *NPHS2* founder mutation R138Q (B) was associated with early onset (1.77 yr; range 0.0 to 5.4 yr). These two groups (A and B) were responsible for the earlier onset of patients with the presence of two *NPHS2* mutations (groups A through D; Table 1). Figure 1A illustrates the significant difference in age at onset of disease between mutation groups A and C ( $P = 0.006$ ). Age of onset for patients with the presence of two other disease-causing *NPHS2* mutations was 5.95 yr (range 0.0 to 14.3 yr) for children with one R138Q and one missense mutation (C). It was 4.17 yr (range 0.0 to 16.6 yr) for children with two missense mutations other than R138Q (D). Both groups (C and D) showed no significant difference compared with patients without disease-causing mutations (E through G; Table 1). The mean age at onset of SRNS was 6.87 yr (range 0.0 to 16.3 yr) for patients with no disease-causing *NPHS2* mutation (E through G) and 6.40 yr (range 0.0 to 21.0 yr) for children free of *NPHS2* changes (H; Table 1). Patients with at least one truncating mutation (nonsense or frameshift) or with homozygous R138Q



**Figure 1.** Kaplan Meier survival analysis of time intervals between *NPHS2* mutation groups A (truncating × any), group B (R138Q × R138Q), group C (R138Q × missense), and group D (missense × missense). (A) Age at onset of disease for all four groups. Each mutation group was compared with group A (earliest age of onset). The age at onset of disease between groups A and C was significantly different ( $P = 0.006$ ). (B) The time interval from onset of disease to development of ESRD. Data on development of ESRD were present in one of nine patients in group C; therefore, this group was excluded from the calculation. Each mutation group was compared with group D (shortest time interval). The time interval was not significantly different between the groups.

mutations therefore showed a significantly earlier onset of SRNS than any other ESRD group of *NPHS2* changes. Survival analysis shows that there is no significant difference in the time interval from onset of disease to development of ESRD between different mutation types (Figure 1B).

The three patient groups with a single mutation or the sequence variant R229Q of unknown significance manifested later; however, there was no significant difference of age of onset neither among groups E, F, or G nor when they were compared with 267 individuals without *NPHS2* changes (H). Later onset of SRNS in children with single *NPHS2* mutations could thus not be observed in this cohort of patients manifesting between 0.0 and 21.0 yr of age. The mean age at manifestation for individuals in these groups was as follows (Table 1): Group E, 6.74 yr (range 0.8 to 16.3 yr) for individuals with single mutation and sequence variant R229Q; group F, 8.12 yr (range 1.6 to 14.7 yr) for patients with single mutations only;

and group G, 6.48 yr (range 0.0 to 13.0 yr) for children with one single heterozygous or a homozygous R229Q sequence variant (Table 1).

Seventy-one (91.0%) of 78 patients with two *NPHS2* mutations and clinical data manifested before their ninth birthday. Six of the seven children who had two mutations and were older than 9 yr at diagnosis carried two missense mutations other than R138Q only.

## DISCUSSION

We report here an *NPHS2* genotype–phenotype correlation for age of onset of SRNS in the largest cohort of children who had SRNS and were examined for *NPHS2* mutations published to date. Of 404 families with SRNS included in this study, 73 (18.1%) of 404 carried two disease-causing *NPHS2* mutations, and 5.1% (21 of 404) carried only one single heterozygous mutation. The R229Q sequence variant of unknown significance was present in 9.2% (37 of 404) of families heterozygously and in 0.5% (two of 404) of families homozygously.

The R138Q mutation was found as the most frequent mutation in 42 (57.5%) of 73 families with two disease-causing mutations. This confirms in our large cohort the role of R138Q as a founder mutation as had been stated by Boute *et al.*<sup>3</sup> in the initial description of *NPHS2*. The proportion of children who had SRNS and carried two recessive *NPHS2* mutations was particularly high among children who were younger than 6 yr. Notably, the presence of at least one truncation mutation or of a homozygous R138Q mutation led to strikingly earlier onset (<1.77 yr) compared with all other groups of *NPHS2* mutations (<4.17 yr). These findings will be important for the counseling of affected families and the prognostic evaluation and management of renal replacement therapy in children with SRNS.

Our analysis shows a genotype–phenotype correlation between specific *NPHS2* mutations and age at onset. Nonsense, frameshift (“truncating mutations”), or homozygous R138Q resulted in significantly earlier presentation of SRNS than all other *NPHS2* changes. In patients with these severe mutations, SRNS had started before 6 yr of age in 98.2% of cases. These findings are consistent with a study by Weber *et al.*<sup>13</sup> Our study further shows that children with one truncating mutation (nonsense or frameshift) and one additional R138Q mutation manifest particularly early (mean 1.0 yr; range 0.0 to 5.5 yr; Supplementary Table 1). This finding further supports the idea that R138Q acts as a severe mutation leading to early onset of SRNS. Functional data have shown that podocin with the R138Q mutation is retained in the endoplasmic reticulum and that this condition results in disrupted targeting of nephrin (*NPHS1*) to lipid raft microdomains.<sup>15–17</sup>

Patients with only one R138Q mutation in combination with one missense mutation (group C) and patients with two missense mutations other than R138Q (group D) manifested significantly later than patients with two severe mutations

(groups A and B; Table 1). Only seven children with two *NPHS2* mutations in this study manifested at >9 yr of age. Six of these seven children carried the missense mutation V290M or V180M.<sup>12</sup> The latter had been reported in association with late onset of SRNS by Weber *et al.*<sup>13</sup> Therefore recessive *NPHS2* mutations as a rule do predominantly result in childhood SRNS before the age of 9 yr.

Our data also show that identical mutations may result in onset of SRNS over a spectrum of several years. This was seen best in patients who had homozygous R138Q mutations and presented between 0.0 and 5.4 yr of age. We therefore conclude that specific mutations have predictive value for age at onset of SRNS, but additional modifying factors are involved. One could speculate that single heterozygous mutations in *NPHS1* could act as a modifier of the NS phenotype in children, but this has been described only extremely rarely (three cases,<sup>18</sup> one case,<sup>13</sup> and no cases<sup>14</sup>); therefore, we did not examine patients for *NPHS1* mutations. We investigated the influence of gender on age of presentation in all 73 patients with disease-causing mutations. The mean age at onset of disease in male patients was 2.74 yr; the mean age at onset of disease in female patients was 2.42 yr. These differences were not statistically significant (Supplementary Table 2).

To determine whether ethnic background had an influence on the age of presentation in patients with disease-causing mutations, we calculated mean age at onset of disease for four different ethnic groups: European patients, Central Slavic patients, Turkish patients, and others. The differences in ages among the groups are small, and statistical analysis was not done because most groups were too small to reach significant conclusions.

Given that *NPHS2* is a recessive disease, only the presence of two disease-causing mutations explains the phenotype. It is not unusual, however, that the second mutation may not be detected in mutational analysis in recessive diseases. The clinical significance of single mutations and the *NPHS2* sequence variant R229Q have been discussed in various studies.<sup>18–22</sup> We therefore included single mutations and the sequence variant of unknown significance R229Q in our analysis as separate groups. The mean age at presentation for patients in whom one single *NPHS2* mutation or R229Q was detected (groups E and F) was significantly different neither from patients with two missense mutations other than R138Q (group D) nor from patients without *NPHS2* changes (group H). Our data therefore neither support the idea that single heterozygous mutations result in late onset SRNS nor eliminate this option.

Specifically, the allele frequency of the R229Q sequence variant of unknown significance has been reported as high as 3.9% in healthy individuals.<sup>22</sup> We found this change in 9.2% (37 of 404) of our families in the heterozygous state and in only 0.5% (two of 404) of all families in the homozygous state. R229Q has been discussed as a modifier of SRNS<sup>18–20</sup>; however, its involvement in the development of FSGS seemed inconclusive to other authors.<sup>22</sup> Our study does not allow a conclusion that R229Q should be considered a disease-causing mutation.

Ardiles *et al.*<sup>21</sup> suggested a correlation between *NPHS2* mutations and age of onset of SRNS. Our data put their suggestion on a statistically solid ground with some modification. We conclude that not homozygosity *versus* heterozygosity of *NPHS2* mutations but rather the allelic type of mutations determines the phenotype by onset of NS. We think that, in addition to *NPHS2* mutations, modifying factors would explain the finding that identical mutations lead to presentation at different ages.

## CONCLUSIONS

The presence of two recessive *NPHS2* mutations caused SRNS in 18.1% (73 of 404) families in this study. We demonstrate for the first time statistical significance for early onset of SRNS in children with truncating or homozygous R138Q mutations of *NPHS2* (<1.77 yr) *versus* children with all other sequence variants (>4.17 yr), including recessive *NPHS2* mutations, with single detected *NPHS2* mutations, with isolated R229Q sequence variants, or without *NPHS2* mutations. This finding will be important for the prognostic evaluation and management of renal replacement therapy in children with SRNS. The vast majority (56 [98.2%] of 57) of children with two truncating or homozygous R138Q mutations presented at <6 yr of age. Truncating or homozygous R138Q mutations are frequent pathogenic *NPHS2* mutations. In this study, they were detected in 71.9% (59 of 82) of all families. Our correlation for age of onset in SRNS does not allow a conclusion on a pathogenic significance of the frequent *NPHS2* sequence variant R229Q.

## CONCISE METHODS

### Patient and Data Recruitment

Human subject research was approved by the University of Michigan institutional review board and the ethics commission of the University of Freiburg, Germany. The diagnosis of NS was made by pediatric nephrologists in specialized centers on the basis of published criteria.<sup>23</sup> Patient recruitment for this study was worldwide with predominance of Central European individuals. After informed consent, clinical data were provided by enrolling specialists using a standardized questionnaire (<http://www.renalgenes.org>), which we have described previously.<sup>1</sup> Blood samples of patients with NS were acquired and used for DNA extraction by standard methods.

To evaluate *NPHS2* genotype–phenotype correlations, we analyzed children affected by SRNS only. SRNS was defined according to published criteria by Arbeitsgemeinschaft für Pädiatrische Nephrologie and the International Study of Kidney Disease in Children (ISKDC).<sup>23,24</sup> Families in which affected individuals had shown any response to standard steroid treatment were excluded from this study. All patients were examined for mutations in *WT1* by direct sequencing of exons 8 and 9.

Of 446 families with SRNS, we excluded from our analysis 42 fam-

ilies in which we identified mutations in *NPHS1*, *LAMB2*, or *WT1*. The cohort analyzed for mutations in *NPHS2* thus included 404 families with 430 patients with SRNS (Table 1).

To compare age of onset between different *NPHS2* sequence changes, we defined onset of SRNS as time of first detection of nephrotic-range proteinuria (>40 mg/m<sup>2</sup> per h) or diagnosis of persistent low-grade proteinuria (>4 mg/m<sup>2</sup> per h). Age at onset of SRNS was documented for 392 patients from 367 families and ranged from 0 to 21.0 yr.

When evaluating frequency of mutations, we considered numbers of families, because siblings have identical mutations. When evaluating clinical data, we considered numbers of patients, because siblings may differ in their clinical characteristics (see Table 1). A subgroup of 202 patients (181 families) in this study had been included in the analysis by Ruf *et al.*,<sup>1</sup> in which it was studied under the aspect of steroid responsiveness among children with *NPHS2* mutations, and 89 patients (80 families) have also been studied in an analysis of genetic causes of NSFL.<sup>14</sup>

### Categories of *NPHS2* Mutations

Patients with two recessive mutations (groups A through D) were classified on the basis of the suggestive data by Weber *et al.*<sup>13</sup> as patients with at least one nonsense or frameshift mutation in combination with any second mutation (A), patients with the homozygous founder mutation R138Q (B), patients with a single heterozygous R138Q mutation in combination with one different missense mutation (C), and patients with two missense mutations other than R138Q (D; Table 1).

The phenotypic relevance of single *NPHS2* mutations and sequence variants of unknown significance in general and the frequent sequence variant R229Q in particular have been discussed by several authors as being involved in the pathogenesis of SRNS.<sup>18–22</sup> Because the pathogenic relevance of these findings has not yet been demonstrated conclusively, we also included single detected mutations and the R229Q sequence variant of unknown significance as separate categories (E through G). We subclassified patients with one heterozygous *NPHS2* mutation in combination with sequence variant R229Q (E), patients with one heterozygous *NPHS2* mutation only (F), and patients with one heterozygous or a homozygous sequence variant R229Q only (G; Table 1). Patients without *NPHS2* mutations or sequence variant R229Q were defined as group H.

### Mutation Analysis of *NPHS2* by Direct Sequencing

Genomic DNA was isolated from blood samples using the Puregene DNA purification kit (Gentra, Minneapolis, MN) following the manufacturer's guidelines. Mutation analysis was performed by exon-flanking direct sequencing of all eight exons of *NPHS2*.<sup>1</sup> Exon primers are available from the authors. For sequence analysis, the software SEQUENCHER (Gene Codes, Ann Arbor, MI) was used. For all detected mutations and other sequence variants, sequencing of both strands was performed. Segregation of these changes was confirmed by direct sequencing of parental samples when available. The absence of previously unpublished mutations was shown in 160 control chromosomes from healthy individuals of matched ethnic origin.

## Statistical Analysis

We tested for differences of mean values of age of onset using a *t* test and considered  $P < 0.05$  significant. Range of age of onset is given in years.

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## DISCLOSURES

None.

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