DOCTORAL THESIS

# MOLECULAR STUDY OF IDIOPATHIC NEPHROTIC SYNDROME

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# RESULTS

# STUDY I

# *HLA-DQA1* and *PLA2R1* Polymorphisms and Risk of Idiopathic Membranous Nephropathy

**SUMMARY**

**Background**

SNPs within *PLA2R1* (rs4664308) and *HLA-DQA1* (rs2187668) were identified as strong risk factors for IMN. PLA2R1 is the major antigen in IMN while HLA-DQA1 plays a central role in immune system response. CNVs within *FCGR3A* and *FCGR3B* genes are associated with various autoimmune diseases due to the crucial role of these genes in the generation of a well-balanced immune response. The extremely variable clinical course of IMN includes spontaneous remission, immunosuppressive therapy response, and poor response to immunosuppressive therapy with progression to ESRD.

**Aims**

The aims of this study were 1) to validate the associations of *HLA-DQA1* and *PLA2R1* risk alleles with IMN in a Spanish population, 2) to study the putative association of CNVs within *FCGR3A* and *FCGR3B* genes with IMN, and 3) to assess the use of these genetic variants in predicting spontaneous remission, immunosuppressive therapy response, and renal function decline.

**Materials and methods**

A cohort of 89 biopsy-proven IMN patients and 286 age- and sex-matched Spanish controls were considered. *PLA2R1* and *HLA-DQA1* SNPs were genotyped using TaqMan SNP Genotyping Assays. Akaike's Information Criterion was used to decide the inheritance model of each SNP. *FCGR3A* and *FCGR3B* CNVs were determined by paralogue ratio test followed by restriction enzyme digest variant ratio assay. Association analyses were performed by means of chi-squared or Fisher's exact test. The contribution of these polymorphisms to predict clinical outcome was analyzed by logistic regression, Kaplan-Meier, and multivariate Cox regression analyses.

**Results**

The association of *HLA-DQA1* (rs2187668) and *PLA2R1* (rs4664308) with IMN was validated in a Spanish cohort. The risk for IMN increased when combining the disease-associated genotypes of both SNPs. No association was found between *FCGR3A* and *FCGR3B* CNVs and IMN. Spontaneous remission was not significantly associated with any of the genetic variants tested, although a higher proportion of *FCGR3B* CNVs was observed in non-spontaneous remission patients. In contrast, carrying the combination of *HLA-DQA1* and *PLA2R1* risk genotypes for IMN development strongly predicted a response to immunosuppressive therapy. *HLA-DQA1*

risk genotypes for IMN development also predicted protection against renal function decline. These predictive values increased when adjusting for baseline proteinuria.

**Conclusions**

The association of *HLA-DQA1* (rs2187668) and *PLA2R1* (rs4664308) with susceptibility of IMN was validated in a Spanish cohort, whereas no significant association was found for *FCGR3A* or *FCGR3B* CNVs. For the first time, evidence is presented for the contribution of these *HLA-DQA1* and *PLA2R1* SNPs to the prediction of IMN immunosuppressive therapy response and renal function decline. This finding may help to identify potential responding and non-responding patients and, thus, provide some help in treatment decisions.

# *HLA-DQA1* and *PLA2R1* Polymorphisms and Risk of Idiopathic Membranous Nephropathy

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## Summary

**Background and objectives** Single nucleotide polymorphisms (SNPs) within HLA complex class II HLA-DQ α-chain 1 (*HLA-DQA1*) and M-type phospholipase A2 receptor (*PLA2R1*) genes were identified as strong risk factors for idiopathic membranous nephropathy (IMN) development in a recent genome-wide association study. Copy number variants (CNVs) within the Fc gamma receptor III (*FCGR3*) locus have been associated with several autoimmune diseases, but their role in IMN has not been studied. This study aimed to validate the association of *HLA-DQA1* and *PLA2R1* risk alleles with IMN in a Spanish cohort, test the putative association of *FCGR3A* and *FCGR3B* CNVs with IMN, and assess the use of these genetic factors to predict the clinical outcome of the disease.

**Design, settings, participants, & measurements** A Spanish cohort of 89 IMN patients and 286 matched controls without nephropathy was recruited between October of 2009 and July of 2012. Case-control studies for SNPs within *HLA-DQA1* (rs2187668) and *PLA2R1* (rs4664308) genes and CNVs for *FCGR3A* and *FCGR3B* genes were performed. The contribution of these polymorphisms to predict clinical outcome and renal function decline was analyzed.

**Results** This study validated the association of these *HLA-DQA1* and *PLA2R1* SNPs with IMN in a Spanish cohort and its increased risk when combining both risk genotypes. No significant association was found between *FCGR3* CNVs and IMN. These results revealed that *HLA-DQA1* and *PLA2R1* genotype combination adjusted for baseline proteinuria strongly predicted response to immunosuppressive therapy. *HLA-DQA1* genotype adjusted for proteinuria was also linked with renal function decline.

**Conclusion** This study confirms that *HLA-DQA1* and *PLA2R1* genotypes are risk factors for IMN, whereas no association was identified for *FCGR3* CNVs. This study provides, for the first time, evidence of the contribution of these *HLA-DQA1* and *PLA2R1* polymorphisms in predicting IMN response to immunosuppressors and disease progression. Future studies are needed to validate and identify prognostic markers.

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## Introduction

Idiopathic membranous nephropathy (IMN) is the most common cause of nephrotic syndrome in the adult white population (1), with an incidence of approximately 1 case per 100,000 persons per year (2). IMN is defined as a histopathological entity characterized by subepithelial deposits of IgG and complement, which causes membrane-like thickening and subsequent proteinuria (3).

The M-type phospholipase A2 receptor (*PLA2R1*) located on podocytes has been identified as the major target antigen, which triggers the accumulation of circulating autoantibodies in more than 75% of individuals with IMN (4,5). Furthermore, autoantibodies against aldose reductase, mitochondrial superoxide dismutase 2, α-enolase and synaptonemal complex protein 65 have been discovered to be present in serum and glomeruli from patients with IMN (6–8). Therefore, IMN is considered to be an autoimmune disease. However, at least six familial cases have been reported, suggesting a genetic contribution to the disease (2,9–13).

Recently, a genome-wide association study involving three independent cohorts (British, Dutch, and French cohorts) identified a highly significant association between IMN and single-nucleotide polymorphisms (SNPs) within *PLA2R1* and HLA complex class II HLA-DQ α-chain 1 (*HLA-DQA1*) genes (14). HLA-DQA1 is part of the heterodimer forming the antigen-binding groove that plays a central role in the immune system by presenting peptides derived from extracellular proteins to immunocompetent cells. Many genes within the *HLA* locus have previously been associated with IMN (15–17). Moreover, other SNPs within *PLA2R1* have been associated with IMN in Taiwanese and Korean populations (18,19). Additional studies to identify and validate genetic risk factors for IMN


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in independent populations may help to elucidate its pathogenesis.

Another important source of genetic variability is copy number variants (CNVs) consisting of gains or losses of DNA segments of at least 1 kb. The Fc gamma receptor (*FCGR*) locus on chromosome 1q23 is subject to CNVs, and their role in susceptibility to various autoimmune diseases has been widely studied (reviewed in ref. 20). The genes included in this locus encode Fc receptors for IgG that have a crucial role in the generation of a well balanced immune response. *FCGR3A* is mainly expressed by natural killer cells and participates in antibody-dependent cell-mediated cytotoxicity, whereas *FCGR3B* is predominantly expressed by neutrophils and is involved in immune complex clearance (21).

The extremely variable clinical course of IMN and the controversial immunosuppressive therapy make treatment decisions challenging (1,22). About one third of IMN patients experience spontaneous remission (SR) of the nephrotic syndrome (23). However, a significant number of patients have a poor response to immunosuppressive therapy and progress to ESRD (24). Prognostic markers of disease progression would be very helpful tools for treatment decision making (25).

The goals of the present study were to (*1*) validate the association of *HLA-DQA1* and *PLA2R1* risk alleles with IMN in a Spanish population, (*2*) study, for the first time, the putative association of CNVs within *FCGR3A* and *FCGR3B* genes with IMN, and (*3*) assess the use of these genetic variants in predicting SR, immunosuppressive therapy response, and decline in renal function.

## Materials and Methods

### Study Population

Spanish patients with biopsy-proven IMN who attended our center between October of 2009 and July of 2012 were recruited ($n$=89). The diagnosis was achieved by renal biopsy performed between 1974 and 2011. None of the patients enrolled had any evidence of a secondary cause of membranous nephropathy. The control group consisted of 286 age- and sex-matched Spanish adults without nephropathy kindly provided by the Biobank of our institution. The study was approved by the Institutional Review Board, and all participants gave their signed informed consent.

For all genotype–phenotype correlation studies, patients referred to our center for renal transplantation ($n$=5) and patients with no clinical information ($n$=1) were excluded (Figure 1). Baseline characteristics and follow-up data of the remaining 83 patients were obtained from medical records until an end point (remission or ESRD) was reached or until July of 2012 (Table 1). Initially, patients were treated using a conservative approach based on supportive treatment with angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers, diuretics, statins, and/or dietary sodium restriction. After an observational period of approximately 6 months, patients with persistent nephrotic syndrome and no significant decrease in proteinuria levels started immunosuppressive therapy. Patients with deterioration of renal function or proteinuria>10 g/d started immunosuppressive therapy at the same time as angiotensin-converting enzyme inhibitors. All patients were treated in our center, and the first-line treatment was based on existing recommendations at that time. In the event of resistance, patients were treated with an alternative immunosuppressive regimen, and in case of relapse, another course of immunosuppressive therapy was attempted.

For the association study of the genetic variants with SR, patients with a minimum follow-up of 2 years were classified according to their clinical outcome into SR or non-SR (NSR) patients, and the latter group was separated into immunosuppressive responders and immunosuppressive nonresponders (Figure 1). Of note, those patients that only received corticosteroid monotherapy ($n$=3) were excluded. SR patients ($n$=23) were defined as achieving partial or complete remission (proteinuria<3.5 or <0.3 g/d, respectively, in at least three consecutive determinations and normal renal function) in the absence of immunosuppressive therapy (23). Responders ($n$=27) included patients treated with one or more courses of immunosuppressive therapy who achieved partial or complete remission. Nonresponders ($n$=28) were defined as patients treated with one or more courses of immunosuppressive therapy who reached ESRD or had no significant and/or sustained reduction of proteinuria levels (proteinuria>3.5 g/d) and severe deterioration of renal function. For renal function decline analysis, the time from renal biopsy to doubling of serum creatinine (DSC) was calculated in 83 patients who had not reached ESRD at diagnosis (Figure 1).

### *HLA-DQA1* and *PLA2R1* SNP Genotyping

Genomic DNA was isolated from peripheral blood using a standard method. SNPs rs2187668 (located within the first intron of the *HLA-DQA1* gene) and rs4664308 (located within the first intron of the *PLA2R1* gene) were genotyped using TaqMan SNP Genotyping Assays (C_58662585_10 and C_27902747_10, respectively) according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Amplification reactions were performed on an ABI 7000 Real-Time PCR System (Applied Biosystems). Internal controls for each genotype were included in all runs. Genotype frequencies for both SNPs were within Hardy–Weinberg equilibrium in controls.

### CNV Analysis

The paralogue ratio test was used to determine CNVs at the *FCGR3* locus (including *FCGR3A* and *FCGR3B* genes). Restriction enzyme digest variant ratio assay was used to distinguish between *FCGR3A* and *FCGR3B* genes based on the work by Hollox *et al.* (26) with small changes (Supplemental Material).

### Statistical Analyses

Descriptive data were expressed as mean±SD for normally distributed variables and median (range) for skewed variables. Comparisons of baseline characteristics among clinical outcome groups were made using Kruskal–Wallis and chi-squared tests. Association analyses were assessed by means of chi-squared or Fisher's exact test when appropriate. SNPStats software was used to decide the best

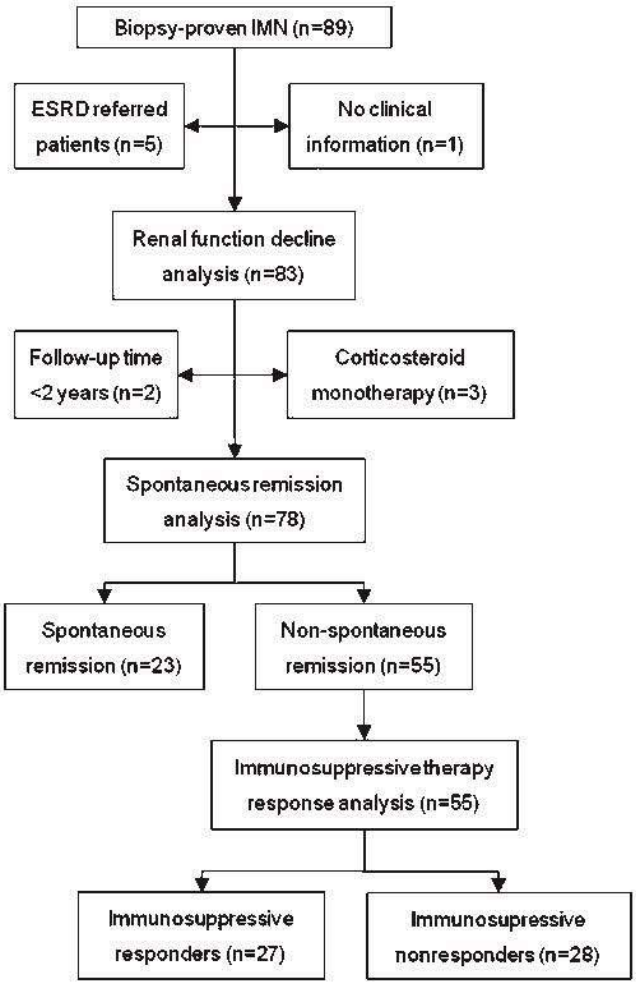


**Figure 1. | Flowchart for the classification of idiopathic membranous nephropathy (IMN) patients included in the genotype–phenotype correlation studies.**

inheritance model for each SNP (27). This software uses the likelihood ratio test to compare every model with the most general model (the codominant) and calculates Akaike's Information Criterion; the best model for a specific SNP is the one with the lowest Akaike's Information Criterion. Unadjusted and adjusted logistic regression analyses were performed to evaluate the relationship between response to immunosuppressive therapy and genetic and clinical variables. Model performance was evaluated using the area under the receiver operating characteristics curve and the Hosmer–Lemeshow goodness-of-fit test. Leave-one-out crossvalidation was performed as an additional measure of accuracy. The odds ratios (ORs) and their 95% confidence intervals (95% CIs) were calculated. Associations between genetic variants and renal survival rate were estimated by the Kaplan–Meier method and the log-rank test. DSC was considered as the primary end point. Multivariate Cox regression analysis was performed to evaluate the relationship between renal function decline and genetic and clinical variables. The hazard ratio was calculated with 95% CI. $P<0.05$ was considered significant for all analyses. Statistical analyses were performed using SPSS version 17.0 software.

## Results

### Association of *HLA-DQA1* and *PLA2R1* SNPs with IMN in a Spanish Cohort

SNPs within *HLA-DQA1* (rs2187668) and *PLA2R1* (rs4664308) genes were genotyped in a Spanish cohort of 89 IMN patients and 286 controls. Association analysis under different genetic models showed that *HLA-DQA1* (rs2187668) and *PLA2R1* (rs4664308) were significantly associated with IMN under a dominant model (OR, 3.70; 95% CI, 2.25 to 6.08; $P<0.001$ and OR, 2.00; 95% CI, 1.23 to 3.23; $P=0.005$, respectively) (Table 2). The risk for IMN increased when combining the disease-associated genotypes of both SNPs (A/A or A/G for *HLA-DQA1* [rs2187668] and A/A for *PLA2R1* [rs4664308]), yielding an OR of 7.33 (95% CI, 3.55 to 15.13; $P<0.001$) (Table 3).

### Association of CNVs of the *FCGR3* Locus with IMN in a Spanish Cohort

The copy number (CN) of *FCGR3A* and *FCGR3B* genes was determined in our Spanish cohort of 89 IMN patients and a subset of 93 controls. The CN profile of *FCGR3A* and *FCGR3B* genes did not differ significantly between IMN patients and controls (Table 4). However, controls

**Table 1. Patients' characteristics according to clinical outcome**

| Patients' Characteristics | Total[a] | Spontaneous Remission | Immunosuppressive Responders | Immunosuppressive Nonresponders | *P* Value |
|---|---|---|---|---|---|
| Number of patients (%) | 83 | 23 (29.5) | 27 (34.6) | 28 (35.9) | |
| Baseline characteristics | | | | | |
| Age (yr) | 46.6±14.4; *n*=83 | 51.0±16.8; *n*=23 | 44.0±12.5; *n*=27 | 46.8±13.6; *n*=28 | 0.37[b] |
| Sex (women/men) | 25/58 | 11/12 | 6/21 | 6/22 | 0.07[c] |
| Proteinuria (g/d) | 5.6 (0.2–20.0); *n*=81 | 2.6 (0.2–15.5); *n*=23 | 6.2 (2.1–18.0); *n*=26 | 6.8 (1.5–20.0); *n*=26 | <0.001[b] |
| Serum creatinine (mg/dl) | 1.0 (0.5–3.8); *n*=81 | 0.8 (0.5–1.9); *n*=23 | 1.0 (0.6–1.9); *n*=27 | 1.15 (0.7–2.7); *n*=27 | 0.01[b] |
| GFR (ml/min per 1.73 $m^2$) | 82 (10–274); *n*=83 | 95 (27–123); *n*=23 | 84 (38–274); *n*=27 | 78 (25–120); *n*=27 | 0.04[b] |
| Disease follow-up | | | | | |
| Follow-up (mo) | 86 (14–420); *n*=83 | 72 (24–346); *n*=23 | 93 (33–255); *n*=27 | 90 (28–324); *n*=28 | 0.52[b] |
| Duration of disease (mo) | 36 (3–283); *n*=77 | 22 (3–50); *n*=23 | 34 (9–183); *n*=27 | 82 (41–283); *n*=15 | <0.001[b] |
| Immunosuppression therapy[d] | | | | | |
| Time to IS therapy (mo) | 6 (0–178); *n*=52 | — | 6 (0–119); *n*=27 | 8 (0–178); *n*=25 | 0.15[b] |
| Ponticelli regimen/CNI/MMF | 7/44/4 | — | 3/24/0 | 4/20/4 | 0.53[c] |
| Rapidity of response (mo) | — | — | 22 (4–116); *n*=27 | — | — |

Data are mean±SD or median (range). GFR was calculated by the Chronic Kidney Disease Epidemiology Collaboration formula. Duration of disease was the time between diagnostic and spontaneous remission/onset of IS-induced remission/ESRD (nonresponsive patients without ESRD were excluded). Time to IS therapy was the time between diagnostics and the start of IS therapy. Rapidity of response was the time between start of IS therapy and onset of remission. IS, immunosuppressive; CNI, calcineurin inhibitor; MMF, mycophenolate mofetil.

[a]Total number considering patients included in the spontaneous remission and IS response analyses (*n*=78) in addition to patients with follow-up times<2 years (*n*=2) and patients treated with corticosteroids monotherapy (*n*=3).

[b]Kruskal–Wallis test among patients with spontaneous remission, IS responders, and IS nonresponders.

[c]Chi-squared test among patients with spontaneous remission, IS responders, and IS nonresponders.

[d]First-line treatment. Patients treated with corticosteroid monotherapy were not considered.

**Table 2. Association between SNPs within *HLA-DQA1* and *PLA2R1* genes and idiopathic membranous nephropathy**

| Gene (SNP) | Ref | MAF | Allelic *P* Value[a] | *n* (Frequency) | | | Codominant Model[b] | | Dominant Model[b,c] | | Recessive Model[b] | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | G/G | A/G | A/A | OR (95% CI); AIC | *P* Value | OR (95% CI); AIC | *P* Value | OR (95% CI); AIC | *P* Value |
| *HLA-DQA1* (rs2187668) | | | | | | | | | | | | |
| IMN | G | 0.287 | <0.001 | 40 (44.9) | 47 (52.8) | 2 (2.2) | 3.88 (2.34 to 6.42); 390.8 | <0.001 | 3.70 (2.25 to 6.08); 389.7 | <0.001 | 1.08 (0.21 to 5.46); 416.7 | 0.92 |
| Controls | | 0.135 | | 215 (75.2) | 65 (22.7) | 6 (2.1) | | | | | | |
| *PLA2R1* (rs4664308) | | | | | | | | | | | | |
| IMN | A | 0.264 | 0.03 | 9 (10.1) | 29 (32.6) | 51 (57.3) | 2.13 (1.27 to 3.57); 410.4 | 0.02 | 2.00 (1.23 to 3.23); 408.9 | 0.005 | 1.12 (0.51 to 2.45); 416.7 | 0.80 |
| Controls | | 0.355 | | 32 (11.2) | 139 (48.6) | 115 (40.2) | | | | | | |

SNP, single-nucleotide polymorphism; *HLA-DQA1*, HLA complex class II HLA-DQ α-chain 1; *PLA2R1*, M-type phospholipase A2 receptor; Ref, reference; MAF, minor allele frequency; OR, odds ratio; 95% CI, 95% confidence interval, AIC, Akaike's Information Criterion; IMN, idiopathic membranous nephropathy.

[a]Chi-squared test.

[b]Likelihood ratio test.

[c]Dominant model was considered A/A and A/G versus G/G (ref) for *HLA-DQA1* and G/G and A/G (ref) versus A/A for *PLA2R1*.

**Table 3. Odds ratios for idiopathic membranous nephropathy according to genotype combinations of SNPs within *HLA-DQA1* and *PLA2R1* genes**

| Genotype Combination | | *n* Cases (%) | *n* Controls (%) | OR (95% CI) | *P* Value[c] |
|---|---|---|---|---|---|
| *HLA-DQA1*[a] (rs2187668) | *PLA2R1*[b] (rs4664308) | | | | |
| G/G | G/G or A/G | 17 (19.1) | 129 (45.1) | 1.00 | |
| G/G | A/A | 23 (25.8) | 86 (30.1) | 2.03 (1.02 to 4.02) | 0.04 |
| A/A or A/G | G/G or A/G | 21 (23.6) | 42 (14.7) | 3.79 (1.83 to 7.86) | <0.001 |
| A/A or A/G | A/A | 28 (31.5) | 29 (10.1) | 7.33 (3.55 to 15.13) | <0.001 |

[a]Classification of *HLA-DQA1* genotypes according to a dominant model.
[b]Classification of *PLA2R1* genotypes considering a dominant model.
[c]Chi-squared test.

showed a trend to low *FCGR3A* CN (5% versus 0%, respectively; *P*=0.06).

### Genotype–Phenotype Correlations

**Genetic Variants and Spontaneous Remission.** We tested whether *HLA-DQA1* (rs2187668), *PLA2R1* (rs4664308), and *FCGR3B* CNVs were associated with IMN SR in a group of 23 SR and 55 NSR patients. The *FCGR3A* gene was not included because of its low variation in CN. No significant association was found for any of these variants. However, all patients who achieved SR (except for one patient) had two copies of the *FCGR3B* gene, whereas 18% of NSR patients presented either high (more than two) or low (less than two) *FCGR3B* CN (Supplemental Table 1).

**Genetic Variants and Immunosuppressive Therapy Response.** Association of these three genetic variants with response to immunosuppressive therapy was assessed by comparing responding (*n*=27) and nonresponding (*n*=28) patients. Genotypes were combined under a dominant model that considered the nonrisk genotypes for IMN susceptibility as a reference. In unadjusted regression analysis, the carriers of the IMN susceptibility genotypes (A/A and A/G for *HLA-DQA1* [rs2187668] or A/A for *PLA2R1* [rs4664308]) showed a trend to response to immunosuppressive therapy that became significant when combining both genotypes (OR, 0.12; 95% CI, 0.02 to 0.72; *P*=0.02). In the multivariate model, adjustment for baseline proteinuria significantly increased the predictive value of this genotype combination for response to immunosuppressive therapy (OR, 0.08; 95% CI, 0.01 to 0.58; *P*=0.01), whereas no association was found for the type of immunosuppressor, age, sex, or baseline serum creatinine (Table 5). This model showed moderate discrimination (area under the receiver operating characteristics curve=0.728; leave-one-out crossvalidation=61.5%), and the Hosmer–Lemeshow test indicated good fit (*P*=0.61). No significant results were found for *FCGR3B* CN (data not shown).

**Genetic Variants and Decline in Renal Function.** Survival analysis over a mean follow-up of 7.2 years of time to DSC was performed considering patients who had not reached ESRD at diagnosis (*n*=83). Results showed that patients carrying the A/A or A/G genotype for *HLA-DQA1* had a longer mean DSC-free time than patients carrying the G/G genotype (16.3 versus 13.0 years, respectively; log-rank *P*=0.05) (Figure 2). Multivariate Cox regression analyses revealed that the A/A and A/G genotypes for *HLA-DQA1* were significant protective factors after adjusting for baseline proteinuria (hazard ratio, 0.37; 95% CI, 0.15 to 0.90; *P*=0.03). No association of the *PLA2R1* SNP or the *FCGR3B* CNVs was found.

## Discussion

In this study, we confirmed the association of *HLA-DQA1* (rs2187668) and *PLA2R1* (rs4664308) with IMN

**Table 4. Association between *FCGR3A* and *FCGR3B* CNVs and idiopathic membranous nephropathy**

| Gene (CNV) | *n* (Frequency) | | | OR (95% CI) | *P* Value[a] |
|---|---|---|---|---|---|
| | CN<2 | CN=2 | CN>2 | | |
| *FCGR3A* | | | | | |
| IMN | 0 (0) | 88 (98.9) | 1 (1.1) | 0.09 (0.01 to 1.65) | 0.06 |
| Controls | 5 (5.4) | 86 (92.5) | 2 (2.2) | | |
| *FCGR3B* | | | | | |
| IMN | 10 (11.2) | 74 (83.2) | 5 (5.6) | 1.66 (0.60 to 4.58) | 0.45 |
| Controls | 7 (7.5) | 81 (87.1) | 5 (5.4) | | |

CNV, copy number variant; CN, copy number; *FCGR3*, Fc gamma receptor III.
[a]Fisher's exact test considering CN<2 versus CN=2 and CN>2.

**Table 5. Logistic regression analyses between SNPs within *HLA-DQA1* and *PLA2R1* genes and idiopathic membranous nephropathy response to immunosuppressive therapy**

| *HLA-DQA1* (rs2187668) | *PLA2R1* (rs4664308) | *n* R (%) | *n* NR (%) | Univariate Analysis[a] OR (95% CI) | Univariate Analysis[a] *P* Value | Multivariate Analysis[a,b] OR (95% CI) | Multivariate Analysis[a,b] *P* Value |
|---|---|---|---|---|---|---|---|
| Genotype | | | | | | | |
| G/G | – | 8 (29.6) | 15 (53.6) | 1.00 | | | |
| A/G | – | 18 (66.7) | 12 (42.9) | 0.37 (0.12–1.11) | 0.08 | 0.32 (0.10–1.00) | 0.05 |
| A/A | – | 1 (3.7) | 1 (3.6) | | | | |
| – | G/G | 3 (11.1) | 2 (7.1) | 1.00 | | | |
| – | A/G | 5 (18.5) | 13 (46.4) | | | | |
| – | A/A | 19 (70.4) | 13 (46.4) | 0.37 (0.12–1.11) | 0.08 | 0.31 (0.09–1.03) | 0.06 |
| Genotype combination | | | | | | | |
| G/G | G/G or A/G | 2 (20.0) | 8 (80.0) | 1.00 | | 1.00 | |
| G/G | A/A | 6 (46.1) | 7 (53.9) | 0.29 (0.05–1.65) | 0.14 | 0.20 (0.03–1.23) | 0.08 |
| A/A or A/G | G/G or A/G | 6 (46.1) | 7 (53.9) | | | | |
| A/A or A/G | A/A | 13 (68.4) | 6 (31.6) | 0.12 (0.02–0.72) | 0.02 | 0.08 (0.01–0.58) | 0.01 |

R, responder to immunosuppressive therapy; NR, nonresponder to immunosuppressive therapy.
[a]Univariate analysis considering a dominant model for *HLA-DQA1* and a dominant model for *PLA2R1*. The nonrisk genotypes for idiopathic membranous nephropathy susceptibility were considered as the reference.
[b]Adjusted for proteinuria at diagnosis.

susceptibility in a Spanish cohort. The combination of high-risk genotypes for both SNPs was associated with higher risk of IMN, which was previously described (14). In contrast, *FCGR3A* and *FCGR3B* CNVs were not significantly associated with IMN. For the first time, we showed that *HLA-DQA1* (rs2187668) and *PLA2R1* (rs4664308) contribute to predict IMN prognosis.

Little is known about the contribution of *HLA-DQA1* and *PLA2R1* genetic variants in IMN pathogenesis. The fact that autoantibody response in IMN is restricted to a conformation-dependent epitope of PLA2R1 led to the hypothesis that modifications in the coding sequence of this gene may contribute to antibody formation (4,28). Coenen *et al.* (29) found no evidence to support this hypothesis;

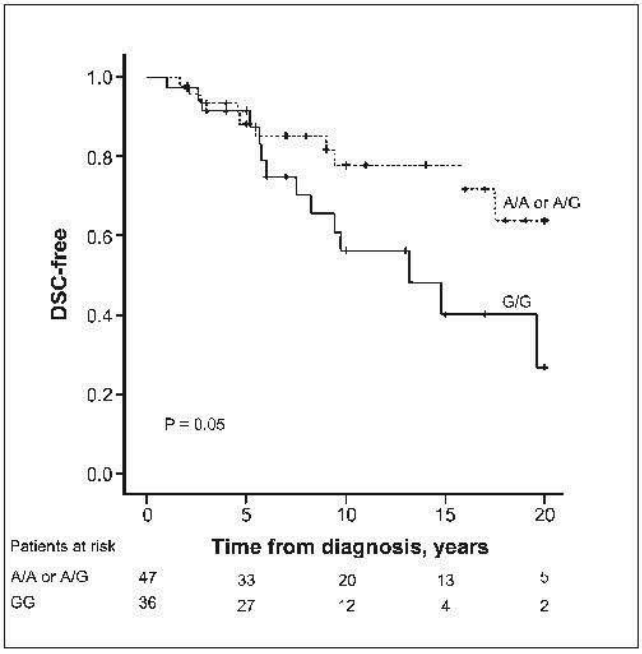


**Figure 2. | Survival analysis of time without doubling serum creatinine (DSC-free) according to the *HLA-DQA1* genotypes.** The number of patients at risk at selected time points is shown below the plot. Log-rank test considering a dominant model for *HLA-DQA1* genotypes shows *P*=0.05.

in a cohort of 95 IMN patients, only 9 patients carried rare sequence variants in the *PLA2R1* gene, and only 4 of the 9 patients were among the 60 patients who presented circulating autoantibodies against PLA2R. Our study provides additional support to the previously found associations between IMN and common coding and noncoding variants within *PLA2R1* and *HLA-DQA1* genes (14,18,19,29). Interestingly, the disease-associated genotype of *PLA2R1* (rs4664308) is the common genotype, which was previously reported (14,30). These associations with relatively common variants, although IMN is a rare disease, raised the hypothesis that the confluence of relatively common polymorphisms in these genes may result in a rare haplotype that confers susceptibility to IMN (29,31).

In this study, we assessed, for the first time, the putative association between *FCGR3A* and *FCGR3B* CNVs and IMN. We hypothesized that low *FCGR3A* CN could decrease antibody-dependent cell-mediated cytotoxicity, thereby playing a protective role in IMN development. Low *FCGR3A* CN was only found in control individuals, supporting our hypothesis; however, statistical significance was not reached. In mice, deletion of the ortholog of human *FCGR3A*, *fcγRIV*, is protective against the development of nephrotoxic nephritis (32). However, in humans, either high or low *FCGR3A* CN was associated with susceptibility to antiglomerular basement membrane disease (33). *FCGR3B* CNVs have been described as a putative risk factor for several autoimmune diseases, such as GN in systemic lupus erythematosus and primary Sjögren's syndrome (34,35). Our results indicate no contribution of *FCGR3B* CNVs to IMN susceptibility. Similarly, no association has been observed in Graves' and Addison's diseases (35,36). Additional studies may help to clarify the relationship between *FCGR3A* and *FCGR3B* CNVs and IMN.

The highly variable clinical course of IMN encourages the search for prognostic markers of clinical outcome. Age at onset<50 years, women, baseline proteinuria<8 g/d, and preserved renal function at presentation are predictors of SR (37,38). The genetic variants analyzed in this study showed no significant association with SR, although 18% of NSR patients exhibited either high (more than two) or low (less than two) *FCGR3B* CN compared with 4% of SR patients; this finding suggests that alterations in *FCGR3B* CN could hinder SR. *FCGR3B* CN was correlated with protein expression and immune complex clearance (39); therefore, changes in *FCGR3B* CN could alter the balance between Fc receptors, disrupting the tightly regulated immune system (20) and impeding achievement of SR.

More interestingly, our results showed that the risk genotypes for IMN development (A/A or A/G for *HLA-DQA1* and A/A for *PLA2R1*) also predict response to immunosuppressive therapy and protection to renal function decline. Recently, Lv *et al*. (30) found that 73% of individuals carrying these IMN susceptibility genotypes had anti-PLA2R antibodies, whereas these antibodies were absent in all carriers of the protective genotypes. In our cohort, immunosuppressive therapy was more effective in patients carrying the IMN susceptibility genotype combination, likely by decreasing anti-PLA2R levels. We speculate that other genetic and environmental factors could contribute to the development of IMN in patients carrying the protective genotypes (G/G for *HLA-DQA1* and A/G or G/G for *PLA2R1*), explaining their low likelihood of response to immunosuppressive treatment. To the best of our knowledge, this association is the first found between genetic variants and clinical outcome in IMN. Thibaudin *et al.* (40) reported an association study of *TNF-α* gene polymorphisms with IMN. This group found a significant association of a SNP in the promoter region and a downstream microsatellite of the *TNF-α* gene with IMN susceptibility. However, no association of these polymorphisms with IMN progression was identified.

We propose that the *HLA-DQA1* (rs2187668) and *PLA2R1* (rs4664308) genotypes could add some predictive value to the currently used clinical and histologic markers. The two most accurate and validated markers for IMN progression to ESRD are the Toronto Risk Score and the urinary excretion of $\beta_2$-microglobulin or IgG (41,42). Recently, the level of anti-PLA2R has been correlated with clinical disease activity (4,5,43,44), and high anti-PLA2R levels have been associated with a significantly reduced frequency of SR (45). The clinical complexity of the disease suggests that a combination of prognostic markers would be the best option for prediction of clinical outcome.

The small size of our cohort is the main limitation of this study. Genotype–phenotype correlation studies require large cohorts of IMN patients with long follow-up time because of their stratification depending on clinical outcome. SR and responder patients attended our center less frequently than nonresponder patients. For this reason, SR and responder patients are underrepresented in our cohort. The inclusion of patients diagnosed over a 30-year period implied the use of different treatment regimens among patients. However, our analysis showed no influence of the type of treatment in the association of *HLA-DQA1* and *PLA2R1* genotypes with immunosuppressive response. Nevertheless, because most patients were treated with calcineurin inhibitors as first-line treatment, our results should be confirmed for patients treated with other immunosuppressive regimens.

In conclusion, we have validated the association of *HLA-DQA1* (rs2187668) and *PLA2R1* (rs4664308) with susceptibility to IMN in a Spanish cohort, whereas no significant association was found for *FCGR3* CNVs. For the first time, we have presented evidence of the contribution of these SNPs to the prediction of IMN response to immunosuppressive therapy and decline in renal function. This finding may help to identify potential responding and nonresponding patients and thus, provide some help in treatment decisions. Future collaborative efforts to incorporate large datasets will indeed be critical to validate the relationship between these genetic variants and IMN clinical outcome.

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**Disclosures**

None.

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**Supplementary methods**

**CNV analysis**

The paralogue ratio test (PRT) was used to determine CNVs at the *FCGR3* locus (including *FCGR3A* and *FCGR3B* genes). The same pair of fluorescently labeled primers amplified the test locus and a region within chromosome 18 known to have two copies (26). We amplified 10 ng of genomic DNA by 24 PCR cycles in order to obtain a non-saturated amount of product using standard conditions. PCR products were differentiated by length using an ABI 3100 Avant Genetic Analyzer (Applied Biosystems, Foster City, CA). Fragment analysis was carried out using Genescan software (Applied Biosystems, Foster City, CA). Peak area ratios between *FCGR3A*+*FCGR3B* (73 bp) and the reference region on chromosome 18 (83 bp) were used to estimate the total copy number (CN) of the *FCGR3* locus. Restriction enzyme digest variant ratio (REDVR) assay was used to distinguish between *FCGR3A* and *FCGR3B* genes using a single fluorescently labeled primer pair (26). Digestion products were separated by capillary electrophoresis on an ABI 3100 Avant Genetic Analyzer (Applied Biosystems Foster City, CA). Peak height ratios between *FCGR3A* (182 bp) and *FCGR3B* (134 bp) were used to estimate the CN of each gene. Mean values for the duplicates were taken. Internal controls for CN<2, CN=2, and CN>2 were run in each experiment.

*FCGR3B* CN was confirmed by duplex quantitative PCR using TaqMan CN assay for *FCGR3B* (Hs04211858_cn, Applied Biosystems) and normalizing with TaqMan Copy Number Reference Assay RNAse P (Part Number 4403326, Applied Biosystems Foster City, CA) on an ABI Prism 7000 Instrument (Applied Biosystems Foster City, CA). Quantitative PCR was carried out with 20 ng of DNA in triplicate following the manufacturer's instructions. Internal controls for CN<2, CN=2, and CN>2 were run in each experiment and CN calculations were performed using the delta-delta Ct method.

**Supplementary Table 1.** Association between SNPs within *HLA-DQA1* and *PLA2R1* genes and *FCGR3B* CNV with spontaneous remission

| Gene (SNP) | | n (Frequency) | | | OR (95% CI) | Genotypic P value |
|---|---|---|---|---|---|---|
| | | G/G | A/G | A/A | | |
| *HLA-DQA1* (rs2187668) | SR | 10 (43.5) | 13 (56.5) | 0 (0) | 0.95 (0.35-2.61) | 0.92[a] |
| | NSR | 23 (41.8) | 30 (54.5) | 2 (3.6) | | |
| *PLA2R1* (rs4664308) | SR | 2 (8.7) | 7 (30.4) | 14 (60.9) | 1.36 (0.48-3.85) | 0.57[a] |
| | NSR | 5 (9.1) | 18 (32.7) | 32 (58.2) | | |
| | | CN <2 | CN =2 | CN >2 | | |
| CN *FCGR3B* | SR | 1 (4.3) | 22 (95.7) | 0 (0) | 4.84 (0.59-39.59) | 0.17[b] |
| | NSR | 7 (12.7) | 45 (81.8) | 3 (5.5) | | |

Abbreviations: SR, spontaneous remission; NSR, no spontaneous remission; CN, copy number; OR, odds ratio; 95% CI, 95% confidence interval.

[a]Genotype frequency difference test ($X^2$) under dominant model for *HLA-DQA1* and *PLA2R1.*

[b]Fisher's exact test considering CN =2 vs CN different from 2.

# STUDY II

# Targeted next-generation sequencing in steroid-resistant nephrotic syndrome: mutations in multiple glomerular genes may influence disease severity

**SUMMARY**

**Background**

The high genetic heterogeneity and phenotypic variability of SRNS makes genetic testing using Sanger sequencing costly and time-consuming. Targeted next-generation sequencing (NGS) of a broad panel of NS-related genes has emerged as a cost-effective strategy to screen the multiple genes involved in SRNS/FSGS.

**Aims**

The goals of this study were to develop a glomerular disease gene panel for genetic diagnosis of SRNS/FSGS and to study the influence of mutations in multiple genes on phenotype variability.

**Materials and methods**

High-throughput mutation analysis of a 26-glomerular-disease-gene panel was performed in a heterogeneous cohort of 50 SRNS/FSGS patients, a validation cohort of 25 patients with previously identified mutations, and a discovery cohort of 25 uncharacterized patients with probable genetic etiology. Five control individuals who had previously been genome-wide genotyped were included to assess the sensitivity and specificity of our targeted NGS panel.

**Results**

In the validation cohort, all 42 previously known pathogenic mutations in their correct heterozygous/homozygous state were detected. Analysis of the controls revealed that our targeted NGS panel had a sensitivity of 95.6% and a specificity of 99.9%. In the discovery cohort, disease-causing mutations in NS genes were identified in 9 out of the 25 SRNS/FSGS patients. Also, three patients carrying mutations in an SRNS/FSGS gene in combination with *COL4A3* were identified. In the clinical phenotype of these patients, the co-existence of NS and microhematuria at presentation stands out. Two of them were familial cases that presented a more severe phenotype than their family members with mutations in only one gene.

**Conclusions**

In this study, the feasibility and robustness of targeted NGS for genetic diagnosis of SRNS/FSGS is demonstrated. This approach allows for a more complete characterization of patients with SRNS/FSGS. Our results indicate that patients carrying mutations in an SRNS/FSGS gene and also in *COL4A3* gene have increased disease severity.

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ARTICLE

# Targeted next-generation sequencing in steroid-resistant nephrotic syndrome: mutations in multiple glomerular genes may influence disease severity

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**Genetic diagnosis of steroid-resistant nephrotic syndrome (SRNS) using Sanger sequencing is complicated by the high genetic heterogeneity and phenotypic variability of this disease. We aimed to improve the genetic diagnosis of SRNS by simultaneously sequencing 26 glomerular genes using massive parallel sequencing and to study whether mutations in multiple genes increase disease severity. High-throughput mutation analysis was performed in 50 SRNS and/or focal segmental glomerulosclerosis (FSGS) patients, a validation cohort of 25 patients with known pathogenic mutations, and a discovery cohort of 25 uncharacterized patients with probable genetic etiology. In the validation cohort, we identified the 42 previously known pathogenic mutations across *NPHS1*, *NPHS2*, *WT1*, *TRPC6*, and *INF2* genes. In the discovery cohort, disease-causing mutations in SRNS/FSGS genes were found in nine patients. We detected three patients with mutations in an SRNS/FSGS gene and *COL4A3*. Two of them were familial cases and presented a more severe phenotype than family members with mutation in only one gene. In conclusion, our results show that massive parallel sequencing is feasible and robust for genetic diagnosis of SRNS/FSGS. Our results indicate that patients carrying mutations in an SRNS/FSGS gene and also in *COL4A3* gene have increased disease severity.**

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## INTRODUCTION

Nephrotic syndrome (NS) is characterized by heavy proteinuria, hypoalbuminemia, edema, and dyslipidemia. Although most patients are steroid-sensitive NS (SSNS), about 20% of children and 40% of adults are steroid-resistant NS (SRNS) and progress to end-stage renal disease (ESRD). In these cases, renal histology typically shows focal segmental glomerulosclerosis (FSGS).[1–3]

Inherited structural defects in the glomerular filtration barrier proteins are responsible for a significant proportion of SRNS.[4,5] Patients with SRNS of genetic origin have poor renal survival but low rate of disease recurrence after renal transplantation.[6] Genetic forms of SRNS can be inherited as an autosomal recessive (AR) or autosomal dominant (AD) condition and can be isolated or syndromic.[5] Mutations in nephrin (*NPHS1*)[7] and podocin (*NPHS2*),[8] with an AR inheritance, are the major cause of congenital and childhood onset NS, respectively. However, mutations in other genes have also been reported.[5,9] Mutations in inverted formin-2 (*INF2*),[10] transient receptor potential channel 6 (*TRPC6*),[11] and rarely, in α-actinin-4 (*ACTN4*)[12] and CD2-associated protein (*CD2AP*)[13] genes cause juvenile or adult onset FSGS with AD inheritance. In rare cases, recessive mutations in *NPHS2* are associated with adult onset FSGS.[14] *De novo* heterozygous mutations in exons 8 and 9 of Wilms tumor (*WT1*) gene can cause both syndromic[15] and isolated childhood onset SRNS.[16] The study of the relative frequency of mutations in the most commonly altered genes in patients with SRNS and/or FSGS allowed the development of genetic testing algorithms based on age at onset, family history, or renal histology.[17–20] However, the genetic heterogeneity and significant phenotypic variability of SRNS make genetic testing using standard Sanger methods costly and time consuming, even if the analysis is restricted to the most frequently mutated genes.

Massive parallel next-generation sequencing (NGS) technology has dramatically increased the throughput and reduced the cost per nucleotide sequenced compared with traditional Sanger methods, enabling cost-effective sequencing of multiple genes simultaneously. Over the past 3 years, whole-exome sequencing has revealed new genes associated with SRNS in a few cases, expanding the genetic heterogeneity of the disease.[21–25] Based on this scenario, targeted NGS

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of a broad panel of NS-related genes has emerged as a cost-effective strategy to screen the multiple genes involved in SRNS/FSGS,[26] but optimal sensitivity and specificity must be demonstrated for each gene in the panel.

In this study, we used targeted NGS to simultaneously sequence 26 genes associated with inherited glomerular diseases in a heterogeneous cohort of 50 SRNS/FSGS patients and 5 control individuals. We aimed to develop a glomerular disease gene panel for SRNS/FSGS and to study the influence of mutations in multiple genes on phenotype variability.

## MATERIALS AND METHODS

### Patients

A total of 50 Spanish patients with idiopathic SRNS/FSGS were included. Patients developing steroid resistance at a later stage of the disease or with recurrence after kidney transplantation were excluded as we considered that they likely had an immunological cause. Biopsy findings included FSGS, minimal change disease (MCD) or diffuse mesangial sclerosis. The validation cohort consisted of 25 patients with known pathogenic mutations in the five most commonly mutated SRNS/FSGS genes that had been previously identified by Sanger sequencing.[18] The discovery cohort consisted of 25 patients with diagnosis of SRNS/FSGS, 21 genetically uncharacterized, and 4 incompletely characterized. All 25 had a probable genetic etiology, based on early onset of the disease ($n=10$), familial history of SRNS/FSGS ($n=11$), or consanguinity ($n=4$). Four of these patients had been analyzed by Sanger sequencing for the most frequently mutated SRNS/FSGS genes in our previous study, and only one recessive pathogenic mutation was identified.[18] We also included five control individuals without nephropathy who had been previously genome-wide genotyped with a HumanOmni 2.5–8 BeadChip (Illumina, Inc., San Diego, CA, USA) to test the performance of the assay across the whole panel. Blood samples were obtained from other family members if they were available. All the samples were codified, and data analysis was performed blindly. The study was approved by the Institutional Review Board, and all participants gave their signed informed consent.

### Sequencing and data analyses

We selected 26 genes associated with hereditary glomerular diseases based on published literature (Table 1). The complete genomic sequence (plus 1 kb of 5′ and 3′ flanking genomic regions) of *NPHS1*, *NPHS2*, *WT1*, *TRPC6*, *INF2*, *LAMB2*, *COL4A3*, *COL4A4*, *COL4A5*, and *GLA* genes and all exons and intron boundaries (plus 100 bp at each end) of the remaining genes were captured using a custom NimbleGen SeqCap EZ Choice Library (Roche NimbleGen, Madison, WI, USA). After removal of repetitive sequences, 83.6% of the targeted bases were covered with capture baits ranging from 68 to 6689 bp (average 1062 bp), for a final targeted region of 0.9 Mb.

Genomic DNA was isolated from peripheral blood using the salting-out method. Libraries were prepared with the TruSeq DNA Sample Preparation Kit (Illumina, Inc.) according to the manufacturer's instructions. In familial cases, only the proband was analyzed by NGS. Pools of 24 individuals were prepared, hybridized to the custom NimbleGen SeqCap EZ Choice Library (Roche NimbleGen) for 72 h, stringently washed, amplified 17 PCR cycles, and run in a HiSeq2000 instrument (Illumina Inc.).

Data analysis was performed blindly with an in-house developed pipeline previously described.[27] All candidate variants were required on both sequenced DNA strands and to account for ≥20% of total reads at that site. Common polymorphisms (≥5% in the general population) were discarded by comparison with dbSNP 138, the 1000G (http://www.1000genomes.org), the Exome Variant Server (http://evs.gs.washington.edu), and an in-house exome variant database to filter out both common benign variants and recurrent artifact variant calls. To identify large structural variants, we used Pindel,[28] Conifer,[29] and PeSV-Fisher (http://gd.crg.eu/tools).

### Evaluation of the pathogenicity of the variants

Nonsense, frameshift, and canonical splice site variants were classified as definitely pathogenic mutations (mutation group (MG) = A). Missense variants were considered *a priori* unclassified sequence variants (UCV), and their potential pathogenicity was evaluated using an *in silico* scoring system developed for the *PKD1* and *PKD2* genes.[30] This scoring system with some minor modifications was tested using previously described pathogenic mutations, for which functional studies had been performed, as positive controls, and known neutral variants or polymorphisms as negative controls.[31–33] This scoring system takes into consideration the biophysical and biochemical difference between wild type and mutant amino acid, the evolutionary conservation of the amino-acid residue in orthologs,[34] a number of *in silico* predictors (Sift, Polyphen, Mutation taster, and Condel), and population data. All candidate pathogenic variants not previously identified were validated by conventional PCR amplification and Sanger sequencing and were not detected in 284 control chromosomes. Segregation of these changes with the disease was assessed for all the available family members. We scored each of these factors, and their sum resulted in an overall variant score (VS). The UCV were classified into four MGs: highly likely pathogenic ($VS \geq 11$, MG = B), likely pathogenic ($5 \leq VS \leq 10$, MG = C), indeterminate ($0 \leq VS \leq 4$, MG = I), and highly likely neutral ($VS \leq -1$, MG = NV). To evaluate the pathogenicity of non-canonical splice site variants, RNA analysis was performed by RT-PCR and Sanger sequencing. If no RNA was available, these variants were analyzed using Alamut version 2.3 (Interactive Biosoftware, Rouen, France), a software package that uses different splice site prediction programs to compare the normal and variant sequences for differences in potential regulatory signals.[35]

We designated pathogenic mutations to be: (i) those sequence variants predicted to result in a truncated protein (MG = A), (ii) canonical and non-canonical splice site variants showed to alter splicing patterns (MG = A), and (iii) those amino-acid substitutions expected to severely alter the protein sequence using *in silico* predictors (MG = B). Missense substitutions classified as MG = C or MG = I were considered as mild mutations in *NPHS1*[32] or variants of unknown clinical significance. All the variants were entered in the Leiden Open Variation Database (http://databases.lovd.nl/shared/genes).

## RESULTS

### Validation of the technology

Sequencing of the 26 glomerular disease gene panel (Table 1) in 50 patients with SRNS/FSGS and 5 control individuals generated a mean of 14.3 million reads per patient. On average, 99.1% of these reads mapped to the reference genome. A mean depth of coverage of 466× was achieved for the 26 targeted genes across all individuals, with 99.6% of targeted bases covered by at least 20 reads (Supplementary Table S1).

The validation cohort included 25 SRNS/FSGS patients who carried a total of 42 known pathogenic mutations in *NPHS1*, *NPHS2*, *WT1*, *TRPC6*, or *INF2* genes and with different phenotypic characteristics (Table 2). We identified all known pathogenic mutations (33 different) in their correct heterozygous/homozygous state, specifically: 22 missense, 3 nonsense, 2 splice site, 4 small deletions, 1 small insertion, and 1 deletion/insertion (Indel) (data not shown). No spurious pathogenic mutations were found in any of these samples. Prior Sanger sequencing of these patients had revealed a total of 285 variants in these genes, 281 of which were also detected by NGS, resulting in 98.6% accuracy.

To assess the sensitivity and specificity of our assay across all 26 genes included in the panel, we evaluated 5 control individuals without nephropathy who had been previously genome-wide genotyped. Sensitivity of detecting homozygous and heterozygous polymorphisms across the 26 genes was 95.6% (1315/1375), and specificity of detecting non-variant sites from the reference genome was 99.9% (3387/3391). No spurious pathogenic mutations were found in any of these samples. Detailed quality control parameters are provided in Supplementary Table S2.

### Sequence variants in NS genes in the discovery cohort

We identified disease-causing mutations in NS genes in 9 out of the 25 SRNS/FSGS patients in the discovery cohort (Table 3).

**Table 1 Panel of genes involved in inherited glomerular diseases**

| *Gene* | *Disease association* | *Inheritance* | *Target* | *Accession no.* | *Chromosome* |
|---|---|---|---|---|---|
| *NPHS1* | CNS, SRNS | AR | Whole gene | NM_004646.2 | 19 |
| *NPHS2* | CNS, SRNS | AR | Whole gene | NM_014625.2 | 1 |
| *WT1* | SRNS, Denys–Drash syndrome | AD | Whole gene | NM_000378.4 | 11 |
| *INF2* | SRNS, FSGS | AD | Whole gene | NM_001031714.3 | 14 |
| *TRPC6* | SRNS, FSGS | AD | Whole gene | NM_004621.5 | 11 |
| *LAMB2* | SRNS, Pierson syndrome | AR | Whole gene | NM_002292.3 | 3 |
| *COL4A5* | Collagen type IV nephropathy | XL | Whole gene | NM_000495.4 | X |
| *COL4A3* | Collagen type IV nephropathy | AD/AR | Whole gene | NM_000091.4 | 2 |
| *COL4A4* | Collagen type IV nephropathy | AD/AR | Whole gene | NM_000092.4 | 2 |
| *GLA* | Fabry disease | XL | Whole gene | NM_000169.2 | X |
| *PLCE1* | CNS, SRNS | AR | Exons | NM_016341.3 | 10 |
| *ACTN4* | SRNS, FSGS | AD | Exons | NM_004924.4 | 19 |
| *CD2AP* | SRNS | AD/AR | Exons | NM_012120.2 | 6 |
| *MYO1E* | SRNS | AR | Exons | NM_004998.3 | 15 |
| *ARHGAP24* | NS, FSGS | AD | Exons | NM_001025616.2 | 4 |
| *CUBN* | NS | AR | Exons | NM_001081.3 | 10 |
| *CFH* | NS | AR | Exons | NM_000186.3 | 1 |
| *COQ2* | NS | AR | Exons | NM_015697.7 | 4 |
| *COQ6* | NS | AR | Exons | NM_182476.2 | 14 |
| *ITGA3* | NS | AR | Exons | NM_002204.2 | 17 |
| *LMX1B* | NS, FSGS | AR | Exons | NM_001174146.1 | 9 |
| *NEIL1* | NS | AR | Exons | NM_001256552.1 | 15 |
| *PDSS2* | NS | AR | Exons | NM_020381.3 | 6 |
| *PTPRO* | SRNS | AR | Exons | NM_030667.2 | 12 |
| *SCARB2* | NS | AR | Exons | NM_005506.3 | 4 |
| *SMARCAL1* | NS | AR | Exons | NM_001127207.1 | 2 |

Abbreviations: AD autosomal dominant; AR, autosomal recessive; CNS, congenital nephrotic syndrome; FSGS, focal segmental glomerulosclerosis; NS, nephrotic syndrome; SRNS, steroid-resistant nephrotic syndrome; XL, X-linked.

**Table 2 Overview of genotypic data obtained by next-generation sequencing**

| | *Total* | *Familial* | *Sporadic* | *Congenital onset* | *Early or late childhood onset* | *Adolescent or adult onset* |
|---|---|---|---|---|---|---|
| *Validation cohort* | 25 | 10 | 15 | 10 | 9 | 6 |
| Patients with pathogenic mutations in an SRNS/FSGS gene | 23 | 9 | 14 | 9 | 8 | 6 |
| Patients with mutations in an SRNS/FSGS gene and *COL4A3* | 2 | 1 | 1 | 1 | 1 | 0 |
| Patients with no pathogenic mutations found | 0 | 0 | 0 | 0 | 0 | 0 |
| *Discovery cohort* | 25 | 15 | 10 | 5 | 12 | 8 |
| Patients with pathogenic mutations in an SRNS/FSGS gene | 9 | 4 | 5 | 5 | 2 | 2 |
| Patients with mutations in an SRNS/FSGS gene and *COL4A3* | 1 | 1 | 0 | 0 | 0 | 1 |
| Patients with no pathogenic mutations found | 15 | 10 | 5 | 0 | 10 | 5 |

Abbreviations: FSGS, focal segmental glomerulosclerosis; SRNS, steroid-resistant nephrotic syndrome. Onset was classified as follows: congenital, 0–3 months; early childhood, 4 months to 5 years; late childhood, 6–12 years; adolescent, 13–18 years; adult, >18 years.

The distribution of mutations in SRNS/FSGS genes differed depending on the age at onset. The mutation detection rate decreased as the age at onset of NS increased. In congenital onset patients (from 0 to 3 months), all the five patients (100%) carried mutations in *NPHS1* ($n=3$) and *NPHS2* ($n=2$) genes. In the early-childhood onset cohort (from 4 months to 5 years), two out of the nine patients (22%) had mutations in *NPHS1* ($n=1$) and *WT1* ($n=1$). No disease-causing mutations were found in any of the three patients with late-childhood onset NS (from 6 to 12 years). In patients with adult onset of NS or FSGS (>18 years), two out of the eight patients (25%) carried mutations in *INF2* ($n=1$) and *TRPC6* ($n=1$) (Table 2). A detailed scoring matrix for the missense variants is provided in Supplementary Table S3.

In the discovery cohort, we included four cases (one familial and three sporadic), with only one recessive pathogenic mutation previously identified by Sanger sequencing. The NGS approach detected variants predicted to alter the non-canonical splice site sequences by the Alamut software but with uncertain clinical significance in three patients.

### Phenotypic effect of mutations in multiple glomerular genes

We found four patients belonging to the validation cohort with three mutated alleles in two recessive SRNS/FSGS genes (Supplementary Table S4). Phenotype modification of the third mutated allele could not be assessed in these patients as three of them were sporadic cases,

**Table 3 Clinical and genetic data of patients in the discovery cohort with disease-causing mutations in an SRNS/FSGS gene and patients with mutations in an SRNS/FSGS gene and *COL4A3***

| Patient | Gender | Familial/sporadic | Age at onset (years) | Features at presentation | Renal biopsy | Immunosuppressive therapy | Evolution | Gene | Mutation 1 (MG) | Mutation 2 (MG) | Gene | Mutation (MG) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Patients in the discovery cohort with disease-causing mutations in an SRNS/FSGS gene* | | | | | | | | | | | | |
| 319 | M | Sp | 0 | CNS | NP | — | Dead at 1 year | *NPHS1* | c.468C>G p.(Y156*) (A) | c.3478C>T p.(R1160*) (A) | | |
| 336 | M | Sp | 0 | CNS | CNF | — | ESRD at 2 months | *NPHS1* | c.1655C>A p.(A552D) (B) | c.1655C>A p.(A552D) (B) | | |
| 299 | F | Sp | 0.1 | CNS | NP | — | ESRD at 8 months | *NPHS1* | c.3250dup p.(V1084Gfs*12) (A) | c.3250dup p.(V1084Gfs*12) (A) | | |
| 324 | M | Sp | 0.3 | NS without edema | DMS | Cs, CP, CsA, MMF − | Normal Cr at 19 years | *NPHS1* | c.1930+5G>A p.(V634Tfs*13) (A) | c.1930+5G>A p.(V634Tfs*13) (A) | | |
| 363 | F | Sp | 0 | CNS | NP | — | Cr 0.37 mg/dl at 1 month | *NPHS2* | c.413G>C p.(R138P) (B) | c.413G>A p.(R138Q) (B) | | |
| 330 | F | Fam[a] | 0.2 | Nephrotic proteinuria, MAL | DMS | — | Normal Cr at 4 months | *NPHS2* | c.320T>C p.(L107P) (B) | c.320T>C p.(L107P) (B) | | |
| 320 | F | Sp | 3 | Denys–Drash syndrome | FSGS | — | ESRD at 4 years | *WT1* | c.1419T>A p.(H473Q) (B) | — | | |
| 347-1 | M | Fam[b] | 19 | Non-nephrotic proteinuria | FSGS | — | CKD stage II at 20 years | *INF2* | c.658G>A p.(E220K) (B) | — | | |
| 347-2 | F | Fam[c] | 20 | Nephrotic proteinuria | FSGS | Cs, CsA − | ESRD at 29 years | *INF2* | c.658G>A p.(E220K) (B) | — | | |
| 384-1 | F | Fam[b] | 27 | Non-nephrotic proteinuria, MH, MAL | NP | — | Normal Cr at 32 years | *TRPC6* | c.2656G>A p.(E886K) (B) | — | | |
| 384-2 | M | Fam[d] | 30 | MAL | NP | — | Normal Cr at 35 years | *TRPC6* | c.2656G>A p.(E886K) (B) | — | | |
| 384-3 | F | Fam[c] | 55 | Non-nephrotic proteinuria, MH | NP | — | Normal Cr at 63 years | *TRPC6* | c.2656G>A p.(E886K) (B) | — | | |
| *Patients with mutations in an SRNS/FSGS gene and COL4A3* | | | | | | | | | | | | |
| 266[e] | F | Sp | 0 | CNS, MH | NP | — | ESRD at 1 year | *NPHS1*[f] | c.514_516del p.(T172del) | c.3250dup p.(V1084Gfs*12) (A) | *COL4A3* | c.3829G>A p.(G1277S) (B) |
| 10-1[e] | M | Fam[b] | 4 | NS, MH | FSGS | Cs, CsA − | ESRD at 12 years | *NPHS2*[f] | c.274G>T p.(G92C) (B) | c.506T>C p.(L169P) (B) | *COL4A3* | c.4504T>C p.(F1502L) (C) |
| 10-2 | F | Fam[g] | 2 | NS | MCD | Cs, CsA ± | Normal Cr at 18 years | *NPHS2*[f] | c.274G>T p.(G92C) (B) | c.506T>C p.(L169P) (B) | *COL4A3* | — |
| 253-1 | F | Fam[b] | 32 | NS, MH | FSGS* | Cs, CsA − | ESRD at 33 years | *INF2* | c.2065C>T p.(R689W) (I) | | *COL4A3* | c.4028-3C>A p.(V1344_G1385del) (A) |
| 253-2 | M | Fam[h] | 39 | Non-nephrotic proteinuria, MH | FSGS | — | ESRD at 51 years | *INF2* | — | | *COL4A3* | c.4028-3C>A p.(V1344_G1385del) (A) |
| 253-3 | M | Fam[i] | — | — | NP | — | Normal Cr at 61 years | *INF2* | — | | *COL4A3* | c.4028-3C>A p.(V1344_G1385del) (A) |
| 253-4 | F | Fam[j] | U | MH | NP | — | Normal Cr at 52 years | *INF2* | — | | *COL4A3* | c.4028-3C>A p.(V1344_G1385del) (A) |
| 253-5 | F | Fam[c] | — | — | NP | — | Normal Cr at 63 years | *INF2* | c.2065C>T p.(R689W) (I) | | *COL4A3* | — |

Abbreviations: CKD, chronic kidney disease; CNF, congenital nephrotic syndrome of Finnish type; CNS, congenital nephrotic syndrome; CP, cyclophosphamide; Cr, creatinine; Cs, corticosteroids; CsA, cyclosporin A; DMS, diffuse mesangial sclerosis; ESRD, end-stage renal disease; F, female; Fam, familial case; FSGS, focal segmental glomerulosclerosis; FSGS*, mesangioproliferative lesions with FSGS; M, male; MAL, microalbuminuria; MCD, minimal change disease; MG, mutation group; MH, microhematuria; MMF, mycophenolate mofetil; NP, not performed; NS, nephrotic syndrome; Sp, sporadic case; U, unknown.

Therapy effect categories: (−), no response; (±), partial reduction of proteinuria. Mutations on these genes were classified according to Genebank Accession numbers: NG_013356.2, NM_004646.2 and NP_004637.1 (*NPHS1*); NG_007535.1, NM_014625.2 and NP_055440.1 (*NPHS2*); NG_009272.1, NM_024426.3 and NP_077744.3 (*WT1*); NG_027684.1, NM_022489.3 and NP_071934.3 (*INF2*); NG_011476.1, NM_004621.5 and NP_004612.2 (*TRPC6*); NG_011591.1, NM_000091.4 and NP_000082.2 (*COL4A3*). The nomenclature used in this study for the description of sequence variants in DNA and protein is in accordance with the Human Genome Variation Society guidelines and can be found at http://www.hgvs.org/. Mutation groups: A, definitely pathogenic; B, highly likely pathogenic → VS ≥ 11; C, likely pathogenic → 5 ≤ VS ≤ 10. Leiden Open Variation Database proband IDs (following the order of the table from top to bottom): 17906, 17907, 19917, 19413, 17908, 17909, 19916, 18450, 18451, 18452, 18453, and 18844.

[a]Only child of consanguineous parents.
[b]Proband.
[c]Proband's mother.
[d]Proband's brother.
[e]Patients of the validation cohort.
[f]Mutations in these genes were previously known in Sanger sequencing.
[g]Proband's sister.
[h]Proband's father.
[i]Proband's uncle.
[j]Proband's aunt.

and only two siblings, both carrying the three mutated alleles, were identified.

We identified three patients carrying mutations in an SRNS/FSGS gene and also in *COL4A3* (Table 3). Patient 266 carried two *NPHS1* pathogenic mutations, an in-frame deletion and a frameshift, together with a heterozygous missense mutation in *COL4A3*, previously reported by Heidet *et al*.[36] She had a congenital NS presenting with microhematuria and no family history of NS. Patient 10-1 and his affected sister (10-2) both carried compound heterozygous missense pathogenic mutations in *NPHS2* gene, but only the proband 10-1 harbored a heterozygous missense variant in *COL4A3* predicted to be likely pathogenic. Both siblings had early childhood onset of SRNS. Patient 10-1 presented with nephrotic range proteinuria and microhematuria. His renal biopsy revealed FSGS, and he developed ESRD at 12 years. His sibling 10-2 presented with borderline nephrotic range proteinuria but no evidence of microhematuria, renal biopsy showed MCD and she presented normal renal function by the age of 18 years (Figure 1a). Patient 253-1 carried a heterozygous splicing mutation in *COL4A3*, demonstrated to produce exon 46 skipping by RNA analysis and predicted to result in a protein lacking 42 amino acids, in combination with a missense variant in the exon 12 of *INF2*. This novel non-conservative substitution, p.R689W, is located at a highly conservative domain (FH2) in the INF2 protein and scored as highly likely pathogenic, using mutation prediction programs. The arginine in the position 689 is totally conserved in mammals and a basic amino acid in all the species. She presented with SRNS and microhematuria at 32 years, and her renal biopsy showed mesangioproliferative lesions with FSGS. Her renal function rapidly deteriorated, reaching ESRD at 33 years. The *COL4A3* mutation was inherited from her affected father (253-2) who presented with non-nephrotic range proteinuria and hematuria at 39 years. His renal biopsy showed FSGS, and he reached ESRD at 51 years. The *INF2* variant was inherited from her asymptomatic mother (253-5). Two of the proband's uncles carried the *COL4A3* mutation, but they only presented microhematuria at 61 (253-3) and 56 years (253-4) (Figure 1b).

## DISCUSSION

In this study, we show that the simultaneous analysis of 26 genes causative of inherited glomerular diseases allows a more complete and efficient characterization of patients with SRNS/FSGS than traditional Sanger sequencing. In addition, we identified three patients carrying combined mutations in an SRNS/FSGS gene and *COL4A3*, suggesting that mutations in different genes that converge in the glomerular filtration barrier influence disease severity.

In the past years, several genetic testing algorithms for SRNS/FSGS have been developed to help in establishing a prioritization of the genes to be sequenced by Sanger. However, the genetic heterogeneity and phenotypic variability of this disease make this approach expensive and time consuming.[17–20] Recently, two studies used NGS technology to analyze the exons and intron boundaries of 24 genes[26] and 21 genes[37] associated with SRNS. Our gene panel included not only genes related with SRNS/FSGS but also genes involved in other glomerular diseases, as we hypothesized that disease severity could be influenced by mutations in multiple glomerular genes. The identification of all previously known pathogenic mutations and no spurious pathogenic mutations in our validation cohort, as well as the high sensitivity and specificity obtained with the analysis of the previously genotyped controls, demonstrate the suitability of this approach for genetic diagnosis of SRNS/FSGS.

In the discovery cohort, we identified disease-causing mutations in NS genes in 9 out of the 25 patients. All patients carried pathogenic mutations in the most likely mutated NS gene according to their age at disease onset.[18] Interestingly, patient 324 had a congenital onset of the disease but still normal renal function at the age of 19 years. He carried a homozygous splicing mutation (c.1930+5G>A) in *NPHS1* found to produce the deletion of the 31 last nucleotides of exon 14 in the mRNA, which is predicted to result in a truncated protein. The mild phenotype of this patient could be explained, because splicing mutations that do not affect the canonical GT/AG splice sites could allow the coexistence of a certain proportion of wild-type *NPHS1* mRNA with the altered mRNA, as previously suggested.[38] Although mRNA analysis from patient's blood did not confirm this hypothesis, we cannot discard the occurrence of this phenomenon in kidneys (Supplementary Figure S1).

We also included four patients with only one recessive candidate pathogenic mutation in an SRNS gene identified by Sanger sequencing. We hypothesized that these patients would carry a large insertion or deletion or a deep intronic splicing mutation as a second pathogenic mutation. Thus we included the whole genomic sequence of the most frequently mutated genes in glomerular diseases in our NGS gene panel and analyzed the data using specific algorithms to search for structural variants. No clear pathogenic mutation was detected, but only variants in non-canonical splice sites were found in three patients. However, RNA from these patients was not available, and the pathogenicity of these variants could not be assessed.

The phenotypic variability observed in SRNS/FSGS patients bearing mutations in the same gene suggests that modifier genes and environmental factors may have a significant role in the renal presentation and outcome.[4] Evidence of oligogenic inheritance with mutations in genes encoding proteins that converge in common pathomechanistic pathways has been reported in Bardet–Biedl syndrome.[39] In addition, the p.R229Q variant in *NPHS2* gene has been suggested to contribute to proteinuria and ESRD in thin basement membrane nephropathy.[40,41] Recently, modifier genes have been proposed to explain early and severe polycystic kidney disease.[42] McCarthy *et al*[26] described two patients carrying a homozygous mutation in *NPHS1* and a possibly pathogenic variant in *WT1*, who developed a more aggressive disease than a third patient carrying the same mutation in *NPHS1* but without the *WT1* variant. To study the putative role of mutations in multiple glomerular genes on SRNS/FSGS clinical variability, disease severity should ideally be compared among various family members with different genotype combinations. Here, four patients carrying three mutated alleles in two SRNS/FSGS genes were found. Unfortunately, three of them were sporadic cases, and only two affected siblings—both carrying the three mutated alleles—were identified. Therefore, the putative effect of the third variant on disease severity could not be assessed.

We identified three patients carrying mutations in an SRNS/FSGS gene in combination with a heterozygous mutation in *COL4A3* gene. Heterozygous mutations in *COL4A3* and *COL4A4* genes cause the mildest phenotype of collagen type IV (α3α4) nephropathy, also named thin basement membrane nephropathy. This nephropathy is characterized by hematuria and low proteinuria,[43,44] and progression to ESRD has recently been described in 30% of cases.[45] The clinical phenotype of the three patients with combined mutations in an SRNS/FSGS gene and *COL4A3* stands out for the coexistence of NS and microhematuria at presentation. Interestingly, in two of these three cases, several family members with different genotype combinations were available (Figure 1). In both families, patients with mutations in an SRNS/FSGS gene and *COL4A3* had a more severe phenotype than their family members carrying mutations in only one gene. Variable disease penetrance in *INF2*-mutated patients has been reported[46] likely

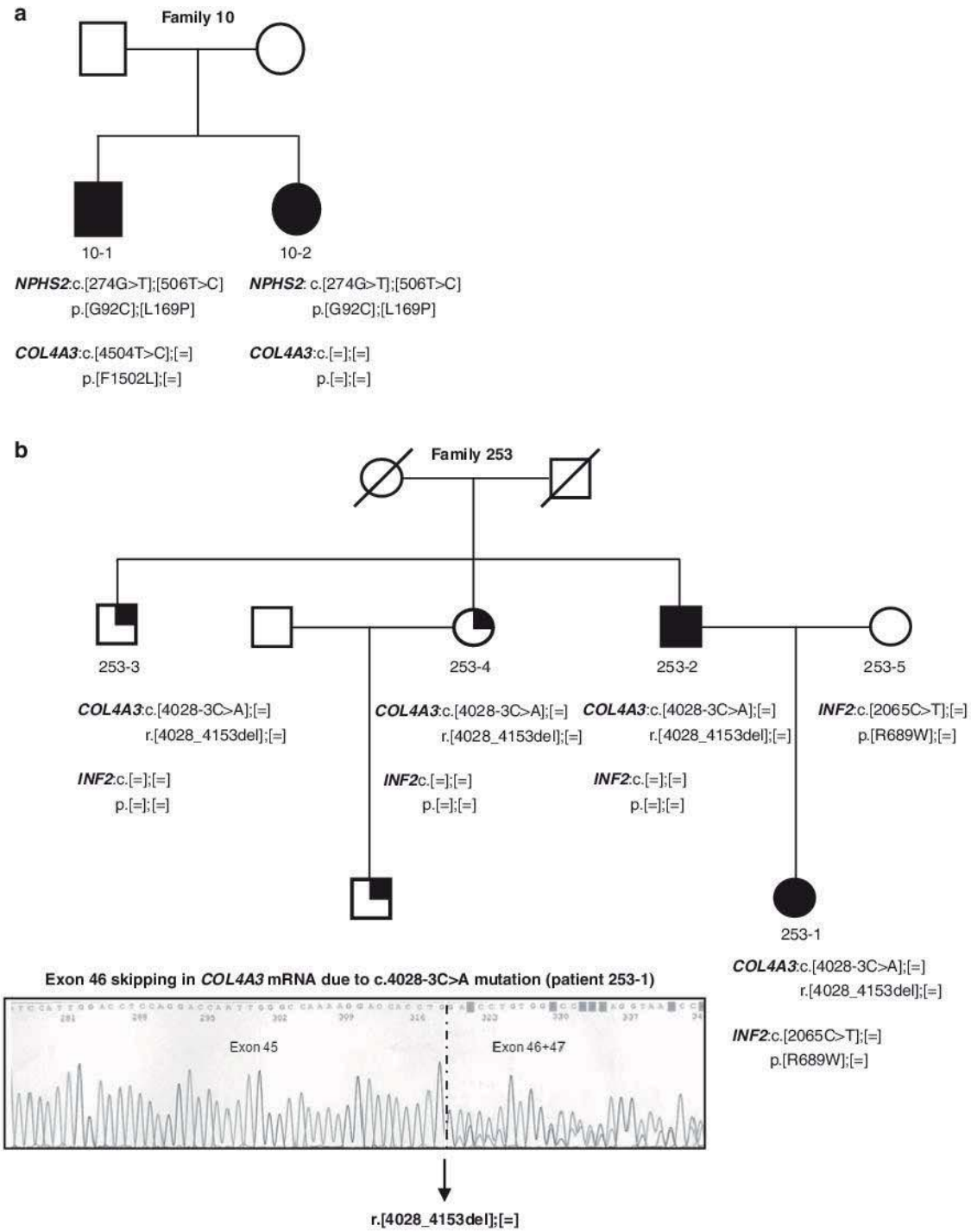


**Figure 1** Pedigrees of two families with mutations in an SRNS/FSGS gene and *COL4A3*. (**a**) In family 10, both siblings had compound heterozygous pathogenic mutations in *NPHS2* gene and the more severely affected individual (10-1) carried an additional likely pathogenic variant in *COL4A3* gene. (**b**) In family 253, individuals 253-1 to -4 carried a pathogenic mutation in *COL4A3* gene demonstrated to produce exon 46 skipping by reverse transcriptase-PCR and Sanger sequencing and predicted to result in a protein lacking 42 amino acids. Patient 253-1 carried an additional variant in *INF2* gene inherited from her mother and developed a more aggressive phenotype than the other affected family members. Cr, creatinine; wt, wild type. The arrows indicate probands. Squares denote males, circles denote females. Filled symbols indicate affected status. Quarter solid symbols indicate microhematuria.

explaining that, in family 253, the proband's mother (253-5) remained asymptomatic. These findings suggest that mutations in multiple glomerular disease genes explain some of the phenotypic variability in nephropathies. Another possible explanation for clinical intrafamilial variability could arise in families carrying a splicing mutation that does not affect the canonical splice sites, such as the mutation in *COL4A3* gene detected in family 253. This mutation could lead to variable amounts of the correctly spliced transcript and could explain the phenotypic variability among the three siblings carrying this splicing mutation.[38]

Despite the broad panel of genes analyzed, we could not find pathogenic mutations in 15 of the patients in the discovery cohort, 8 of whom were familial cases. The fact that some SRNS/FSGS patients present with recurrence after kidney transplantation indicates that some of these cases may be due to an immunological cause, although no evidence of immunological bases was observed in our cohort. In the familial cases, it is highly likely that an SRNS/FSGS gene, as yet non-identified, is responsible for the disease. The next step should therefore be to sequence the whole exome in the 8 familial cases to identify new candidate genes.

The results obtained in the validation cohort demonstrate that our approach is suitable for genetic diagnosis of SRNS/FSGS but, based on the discovery cohort findings, we propose some modifications: (1) to sequence a gene panel with only the six most frequently mutated genes in SRNS/FSGS (*NPHS1*, *NPHS2*, *PLCE1*, *WT1*, *INF2*, *TRPC6*). The *COL4A3*, *COL4A4* and *COL4A5* genes, associated with collagen type IV (α3α4) nephropathy, could also be included as they may influence disease severity. If no pathogenic mutations are identified, a more extensive glomerular gene panel or exome sequencing could be performed; and (2) to restrict the targeted sequence to exons and intron boundaries as the assessment of the pathogenicity of deep intronic variants is challenging and their involvement in the disease speculative. In terms of the cost, NGS will allow the simultaneous analysis of around 250 exons for approximately the same cost of consumables than sequencing 40 exons by Sanger, with three times saving in hands-on time. Identifying pathogenic mutations in SRNS is important for many reasons. It can help to avoid the adverse effects of steroid therapy, modify the intensity and duration of immunosuppressive therapies, encourage living donor kidney transplantation, provide prognostic information regarding the gene and type of mutations, and enable genetic counseling. Sequencing a panel of genes involved in glomerular inherited diseases will also help to elucidate cases with atypical renal phenotypes and/or with high clinical intrafamilial variability. Based on our findings, such cases could be more prevalent than previously expected.

In conclusion, this study shows the feasibility and robustness of targeted NGS for genetic diagnosis of SRNS/FSGS, allowing a more complete characterization of patients with SRNS/FSGS. Our results indicate that patients carrying mutations in an SRNS/FSGS gene and also in *COL4A3* gene have increased disease severity.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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**Supplementary Table 1**. Coverage statistics of the 26-glomerular disease gene panel

| Gene | Target coverage (%) | Mean depth (x) | Depth ≥100x (%) | Depth ≥50x (%) | Depth ≥20x (%) | Depth ≥10x (%) | Depth ≥5x (%) | Depth ≥1x (%) |
|---|---|---|---|---|---|---|---|---|
| ALL | 99.91 | 465.72 | 95.95 | 98.80 | 99.56 | 99.73 | 99.81 | 99.91 |
| *NPHS1* | 100.00 | 590.30 | 98.58 | 99.89 | 99.99 | 100.00 | 100.00 | 100.00 |
| *NPHS2* | 100.00 | 533.42 | 96.88 | 98.09 | 98.83 | 99.44 | 99.82 | 100.00 |
| *WT1* | 99.95 | 512.64 | 97.41 | 98.30 | 99.02 | 99.44 | 99.69 | 99.95 |
| *INF2* | 99.53 | 444.92 | 95.79 | 97.84 | 98.65 | 98.93 | 99.15 | 99.53 |
| *TRPC6* | 100.00 | 397.87 | 96.11 | 99.04 | 99.80 | 99.95 | 99.99 | 100.00 |
| *LAMB2* | 100.00 | 652.95 | 95.97 | 98.58 | 99.79 | 99.97 | 100.00 | 100.00 |
| *COL4A5* | 100.00 | 274.88 | 89.99 | 98.18 | 99.73 | 99.93 | 99.98 | 100.00 |
| *COL4A3* | 100.00 | 447.63 | 96.99 | 99.15 | 99.72 | 99.91 | 99.97 | 100.00 |
| *COL4A4* | 100.00 | 462.38 | 96.49 | 98.77 | 99.54 | 99.79 | 99.92 | 100.00 |
| *GLA* | 100.00 | 358.18 | 96.01 | 99.59 | 99.93 | 99.98 | 100.00 | 100.00 |
| *PLCE1* | 99.62 | 582.17 | 97.81 | 98.45 | 98.66 | 98.86 | 99.09 | 99.62 |
| *ACTN4* | 99.99 | 614.85 | 92.17 | 95.23 | 98.12 | 99.20 | 99.67 | 99.99 |
| *CD2AP* | 100.00 | 268.86 | 89.67 | 97.65 | 99.81 | 99.95 | 99.98 | 100.00 |
| *MYO1E* | 100.00 | 570.37 | 98.49 | 99.73 | 99.96 | 99.99 | 100.00 | 100.00 |
| *ARHGAP24* | 99.98 | 521.64 | 97.55 | 99.19 | 99.76 | 99.87 | 99.90 | 99.98 |
| *CUBN* | 100.00 | 528.75 | 97.62 | 99.32 | 99.91 | 99.99 | 100.00 | 100.00 |
| *CFH* | 99.95 | 357.15 | 91.38 | 97.10 | 98.65 | 98.85 | 99.20 | 99.95 |
| *COQ2* | 99.99 | 343.29 | 87.59 | 94.66 | 98.58 | 99.48 | 99.86 | 99.99 |
| *COQ6* | 100.00 | 645.30 | 99.78 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |
| *ITGA3* | 100.00 | 611.80 | 93.40 | 97.79 | 99.61 | 99.92 | 99.99 | 100.00 |
| *LMX1B* | 98.56 | 471.47 | 90.17 | 92.65 | 94.60 | 95.69 | 96.51 | 98.56 |
| *NEIL1* | 100.00 | 571.56 | 96.69 | 98.93 | 99.90 | 99.98 | 99.99 | 100.00 |
| *PDSS2* | 100.00 | 476.96 | 96.89 | 99.90 | 100.00 | 100.00 | 100.00 | 100.00 |
| *PTPRO* | 100.00 | 540.18 | 97.65 | 99.10 | 99.90 | 99.98 | 100.00 | 100.00 |
| *SCARB2* | 98.75 | 474.95 | 94.12 | 95.24 | 95.97 | 96.60 | 97.19 | 98.75 |
| *SMARCAL1* | 100.00 | 676.42 | 99.27 | 99.93 | 100.00 | 100.00 | 100.00 | 100.00 |

**Supplementary Table 2.** Sequencing quality control parameters of the 26-glomerular disease gene panel in the five control individuals

| | | Control individuals | | | | |
|---|---|---|---|---|---|---|
| | **Sum** | **462** | **586** | **606** | **616** | **624** |
| **Total genotyped positions** | 4766 | 953 | 954 | 953 | 953 | 953 |
| **Total genotyped SNPs** | 1375 | 258 | 276 | 287 | 297 | 257 |
| **Total NGS SNPs** | 1319 | 248 | 264 | 276 | 284 | 247 |
| NGS TP | 1315 | 247 | 263 | 275 | 283 | 247 |
| NGS FP | 4 | 1 | 1 | 1 | 1 | - |
| NGS TN | 3387 | 694 | 677 | 665 | 655 | 696 |
| NGS FN | 60 | 11 | 13 | 12 | 14 | 10 |
| NGS PPV | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| NGS sensitivity | 0.96 | 0.96 | 0.95 | 0.96 | 0.95 | 0.96 |
| **Genotyping Het** | 919 | 191 | 179 | 179 | 187 | 183 |
| Het TP | 886 | 186 | 170 | 174 | 179 | 177 |
| Het TP (correct allele) | 880 | 186 | 167 | 172 | 178 | 177 |
| Het TP (wrong allele) | 6 | | 3 | 2 | 1 | - |
| Het FP | 3 | 1 | 1 | - | 1 | - |
| Het FN | 33 | 5 | 9 | 5 | 8 | 6 |
| Het PPV | 1.00 | 0.99 | 0.99 | 1.00 | 0.99 | 1.00 |
| Het sensitivity | 0.96 | 0.97 | 0.95 | 0.97 | 0.96 | 0.97 |
| **Genotyping Hom** | 456 | 67 | 97 | 108 | 110 | 74 |
| Hom TP | 429 | 61 | 93 | 101 | 104 | 70 |
| Hom TP (correct allele) | 427 | 61 | 93 | 101 | 103 | 69 |
| Hom TP (wrong allele) | 2 | - | - | - | 1 | 1 |
| Hom FP | 0 | - | - | - | - | - |
| Hom FN | 27 | 6 | 4 | 7 | 6 | 4 |
| Hom PPV | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Hom sensitivity | 0.94 | 0.91 | 0.96 | 0.94 | 0.95 | 0.95 |

Abbreviations: FP, false positive; FN, false negative; Het, heterozygous; Hom, homozygous; NGS, next-generation sequencing; PPV, positive predictive value; SNPs, single nucleotide polymorphisms; TN, true negative; TP, true positive.

PPV was calculated as: [number of true positives/(number of true positives + number of false positives)]

**Supplementary Table 3**. Classification of amino acid substitutions (*in silico* scoring system) found in the discovery cohort

| Substitutions | Exon | Previous description[a] | Control Chrs[b] | Segregation analysis[c] | GD[d] | GV[e] | GD/GV matrix score[f] | GDev[g] | Polyphen[h] | SIFT[i] | Condel[j] | Mutation Taster[k] | SNP database[l] | VS | MG[m] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***NPHS1*** | | | | | | | | | | | | | | | |
| E121K | 3 | (1)(+1) | 0/284 (+2) | +4 | 57 | 112.9 | -2 | 36.7 (+1) | +0 | -1 | -1 | -1 | +0 | 3 | **I** |
| R367C | 9 | (2)(+1) | 0/284 (+2) | +4 | 180 | 85.1 | +4 | 126.7 (+0) | +1 | +1 | +1 | +1 | +0 | 15 | **B** |
| A552D | 13 | Novel | 0/284 (+2) | NA | 126 | 0 [xe] | +6 | 125.8 (+2) | +1 | -1 | +1 | +1 | +0 | 12 | **B** |
| C623F | 14 | (3)(+1) | 0/284 (+2) | NA | 204 | 0.0 | +8 | 204.4 (+2) | +1 | +1 | -1 | +1 | +0 | 15 | **B** |
| ***NPHS2*** | | | | | | | | | | | | | | | |
| G92C | 1 | (4)(+1) | 0/284 (+2) | +4 | 158 | 0.0 | +7 | 158.2 (+2) | +1 | +1 | -1 | +1 | +0 | 18 | **B** |
| L107P | 2 | (5)(+1) | 0/284 (+2) | NA | 98 | 51.3 | +3 | 67.8 (+1) | +1 | +1 | +1 | +1 | +0 | 11 | **B** |
| R138P | 3 | (6)(+1) | 0/284 (+2) | NA | 103 | 0.0 | +6 | 102.7 (+2) | +1 | +1 | +1 | +1 | +0 | 15 | **B** |
| R138Q | 3 | (4)(+1) | 0/284 (+2) | NA | 43 | 0.0 | +5 | 42.8 (+2) | +1 | +1 | +1 | +1 | +0 | 14 | **B** |
| L169P | 4 | (7)(+1) | 0/284 (+2) | +4 | 98 | 0.0 | +6 | 97.8 (+2) | +1 | +1 | +1 | +1 | +0 | 19 | **B** |
| ***WT1*** | | | | | | | | | | | | | | | |
| H473Q | 9 | Novel | 0/284 (+2) | +4 | 24 | 0.0 | +2 | 24.1 (+2) | +1 | +1 | +1 | +1 | +0 | 14 | **B** |
| ***INF2*** | | | | | | | | | | | | | | | |
| E220K | 4 | (10)(+1) | 0/284 (+2) | +4 | 57 | 0.0 | +5 | 56.9 (+2) | +1 | +1 | +1 | +1 | +0 | 18 | **B** |
| R689W | 12 | Novel | 0/284 (+2) | -10 | 101 | 26.0 | +5 | 96.0 (+2) | +1 | +1 | -1 | +1 | +0 | 1 | **I** |
| ***TRPC6*** | | | | | | | | | | | | | | | |
| E886K | 13 | Novel | 0/284 (+2) | +4 | 57 | 0.0 | +5 | 56.9 (+2) | +1 | +1 | +1 | +1 | +0 | 17 | **B** |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***PLCE1*** | | | | | | | | | | | | | | | |
| R1195W | 12 | Novel | 0/284 (+2) | NC | 101 | 26.0 | +5 | 95.8 (+2) | +1 | +1 | +1 | +1 | +0 | 13 | **B** |
| H1905R | 26 | Novel | 0/284 (+2) | NC | 29 | 0.0 | +2 | 28.8 (+2) | +0 | -1 | +1 | +1 | +0 | 7 | **C** |
| ***CUBN*** | | | | | | | | | | | | | | | |
| D2160G | 43 | Novel | 0/284 (+2) | NC | 94 | 0.0 | +6 | 93.8 (+2) | +1 | -1 | +1 | +1 | -1 | 11 | **B** |
| ***COL4A3*** | | | | | | | | | | | | | | | |
| G1277S | 43 | (11)(+1) | 0/284 (+2) | NC | 55 | 0.0 | +5 | 55.3 (+2) | +1 | +1 | -1 | +1 | -1 | 11 | **B** |
| F1502L | 49 | Novel | 0/284 (+2) | NC | 22 | 0.0 | +2 | 21.8 (+2) | +1 | +1 | -1 | +1 | +0 | 8 | **C** |
| ***LAMB2*** | | | | | | | | | | | | | | | |
| R94W | 4 | Novel | 0/284 (+2) | NC | 101 | 0.0 | +6 | 101.3 (+2) | +1 | +1 | +1 | +1 | +0 | 14 | **B** |

Abbreviations: Chrs, Chromosomes; NA, not assessed; NC, not considered; MG, mutation group; SNP, single nucleotide polymorphism, VS, variant score; [a]When a change was described previously in the literature as a pathogenic mutation or in the HGM database (+ 1); [b]When a sequence variant was not present in the control chromosomes (+2), if present less than 1% (−1) or more than 1% (-2); [c]Segregation demonstrated in family (+4) if there were affected siblings or an affected parent, (-10) present in asymptomatic patient; [d]GD (Grantham distance), score of chemical difference between the normal and mutated residue (high score, greater difference); [e]GV (Grantham variation), score of chemical difference between orthologs (ranging from chimpanzee to zebrafish, 0 = completely conserved among orthologs, [xe] = conserved among orthologs except in *Xenopus*; [f]GD/GV matrix score, ranging from -2 to +8 [lower matrix scores corresponded to low GD and high GV (conservative change and strong variation within the MSA), while higher matrix scores corresponded to high GD and low GV (non-conservative change and strong conservation within the multiple sequence alignment, MSA)]; [g]GDev (Grantham deviation), score of chemical difference between the mutated residue and the range of variation between orthologs (GD similar to GDev, higher difference, +2); [h]Polyphen prediction: probably damaging (+1); possibly damaging (+0); benign (−1); [i]SIFT prediction: not tolerated (+ 1), tolerated (-1); [j]Condel prediction: deleterious (+1), neutral (-1); [k]Mutation Taster: disease causing (+1), polymorphism (−1); [l]SNP database: not described (+0), described (−1); [m]MG: B, highly likely pathogenic→VS≥11; C, likely pathogenic→5≤VS≤10; I, indeterminate pathogenicity→−4≤VS≤4; NV, neutral variant →VS≤−5.

**Supplementary Table 4.** Clinical and genetic data of patients with three mutated alleles in two NS genes

| Family | Gender | Familial/ sporadic | Age at onset (years) | Features at presentation | Renal biopsy | Immunosuppressive therapy | Evolution | Gene | Mutation 1 (MG) | Mutation 2 (MG) | Gene | Mutation (MG) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 20 | M | Sp | 0 | NS | NP | - | ESRD at 1 years | *NPHS1*[c] | c.1701C>A p.(C567*) (A) | c.1868G>T p.(C623F) (B) | *LAMB2* | c.280C>T p.(R94W) (B) |
| 19 | F | Sp | 0.2 | NS | CNF | - | ESRD at 1 years | *NPHS1*[c] | c.1701C>A p.(C567*) (A) | c.3343G>T p.(E1115*) (A) | *CUBN* | c.6479A>G p.(D2160G) (B) |
| 79-1 | F | Fam[a] | 6 | NS | FSGS | Cs, CsA, CP, MMF - | Normal Cr at 20 years | *NPHS1*[c] | c.1099C>T p.(R367C) (B) | c.361G>A p.(E121K) (I) | *PLCE1* | c.3583C>T p.(R1195W) (B) |
| 79-2 | M | Fam[b] | 1 | NS | FSGS* | Cs, CsA - | Normal Cr at 12 years | *NPHS1*[c] | c.1099C>T p.(R367C) (B) | c.361G>A p.(E121K) (I) | *PLCE1* | c.3583C>T p.(R1195W) (B) |
| 77 | F | Sp | 5 | NS | FSGS | Cs, CP, CsA, MMF - | ESRD at 13 years | *NPHS2*[c] | c.855_856del p.(R286Tfs*17) (A) | c.855_856del p.(R286Tfs*17) (A) | *PLCE1* | c.5714A>G p.(H1905R) (C) |

Abbreviations: CNF, congenital nephrotic syndrome of Finnish type; CP, cyclophosphamide; Cr, creatinine; Cs, corticosteroids; CsA, cyclosporin A; ESRD, end-stage renal disease; F, female; Fam, familial case; FSGS, focal segmental glomerulosclerosis; FSGS*, mesangioproliferative lesions with FSGS; M, male; MG, mutation group; MMF, mycophenolate mofetil; NP, not performed; NS, nephrotic syndrome; Sp, sporadic case.

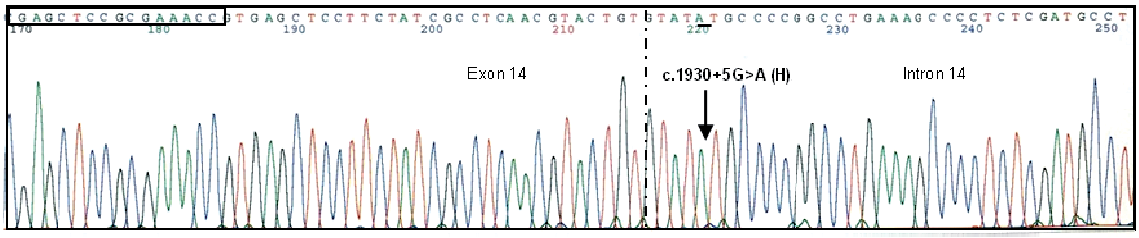
Therapy effect categories: (-) no response. Mutations on these genes were classified according to Genebank Accession numbers: NG_013356.2, NM_004646.2 & NP_004637.1 (NPHS1); NG_007535.1, NM_014625.2 & NP_055440.1 (NPHS2); NG_008967.1, NM_001081.3 & NP_001072.2 (CUBN); NG_008094.1, NM_002292.3 & NP_002283.3 (LAMB2); NG_015799.1, NM_016341.3 & NP_057425.3 (PLCE1). The nomenclature used in this study for the description of sequence variants in DNA and protein is in accordance with the Human Genome Variation Society guidelines and can be found at http://www.hgvs.org/. Mutation groups: A, definitely pathogenic; B, highly likely pathogenic→VS≥11; C, likely pathogenic→5≤VS≤10.

[a]Proband; [b]Proband's brother; [c]Mutations in these genes were previously known by Sanger sequencing.

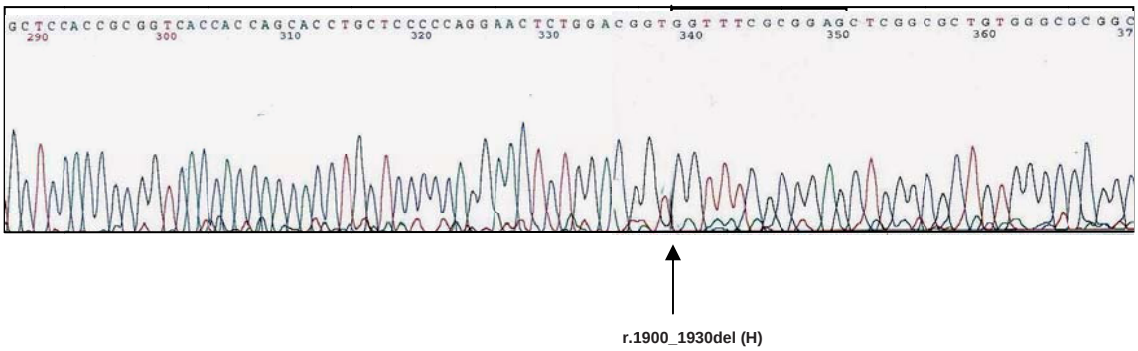
Leiden Open Variation Database proband IDs (following the order of the table from top to bottom): 18845, 19409, 19411, 19412.

**Supplementary Figure 1:** *NPHS1* mutation analysis of patient 324 at a) DNA (c.1930+5G>A) and, b) mRNA (r.1900_1930del31) level, which produces the deletion of the last 31 nucleotides of exon 14. c) Schematic representation of the wild type and abnormal mRNA sequences, the deleted sequence is highlighted. The boxed nucleotides indicate the same sequence in DNA (in forward) and mRNA (in reverse). Abbreviations: homozygous (H).

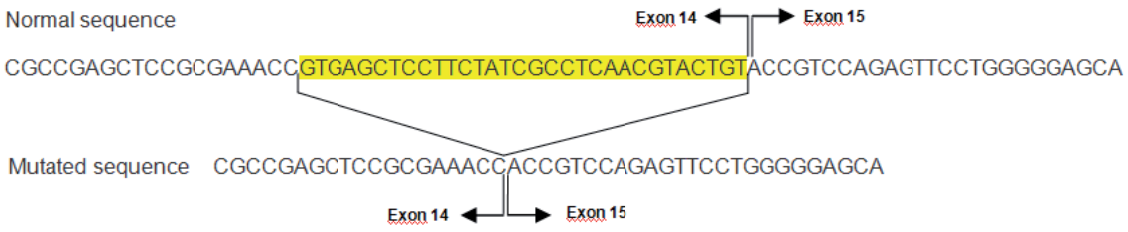
a)




b)




c)



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