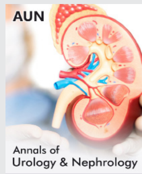

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Research Article

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NPHS1 and *NPHS2* Variants Associated with Early-Onset Frequently Relapsing or Steroid-Dependent Nephrotic Syndrome

Ahmad O Babalghith[1], Nasser A Elhawary[1]*, Iman S Abumansour[1], Ehab M Melibary[1], Ghydda Alghamdi[1], Ikhlas A Sindi[2], Ezzeldin N Elhawary[3], Najiah M Alyamani[4], Ibtesam S Almami[5], Hind Naffadi[6], Afnan Shakoori[7], Luai Zamil[8], Nasser Alenezy[9] and Mohammed Dandini[10]

[1]*Department of Medical Genetics, College of Medicine, Umm Al-Qura University, Mecca, SA*

[2]*Department of Biotechnology, Faculty of Science, King Abdulaziz University, SA*

[3]*MS Genomic Medicine Program, Faculty of Medicine, University of Southampton, Southampton General Hospital, UK*

[4]*Department of Biology, College of Science, University of Jeddah, Jeddah, SA*

[5]*Department of Biology, College of Science, Qassim University, SA*

[6]*Common Science, First Year Deanship, Umm Al-Qura University, Mecca, SA*

[7]*Department of Laboratory Medicine, Faculty of Applied Medical Sciences, Umm Al-Qura University, Mecca, SA*

[8]*Department of Laboratory and Blood Bank, King Abdulaziz Hospital, Mecca, SA*

[9]*Rumah General Hospital, Ministry of Health; Riyadh, SA*

[10]*Department of Laboratory and Blood Bank, Maternity and Children Hospital, Mecca, SA*

***Corresponding author:** Nasser A Elhawary, Department of Medical Genetics, Umm Al-Qura University, Mecca 21955, P.O. 57543, Saudi Arabia.

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Abstract

Background: Glomerular podocytes and slit diaphragms remain the major causes of progressive proteinuria and nephrotic syndrome (NS).

Objective: Here, we analyzed genetic variants in the nephrin (*NPHS1*) and podocin (*NPHS2*) genes in cases with early-onset presentation of NS in Saudi Arabia.

Subjects and methods: Clinical characteristics and genomic DNA were collected from 35 unrelated NS cases (mean age of onset, 3.0 years; range, stillbirth-8 years). Cases were diagnosed based on histologic kidney biopsy and biochemical parameters. Enzymatic digestion was performed on PCR amplicons for the frameshift L41NfsX50 and nonsense R1109X variants of *NPHS1*, and the entire coding regions of *NPHS2* were sequenced.

Results: We identified 15 cases of frequently relapsing NS or steroid-dependent NS (FRNS/SDNS) (42.9%), 12 cases of steroid-resistant NS (SRNS) (34.3%), and 8 cases of steroid-sensitive NS (SSNS) (22.9%). Cases with FRNS/SDNS (46.2%) had a younger age at presentation than cases with SSNS (38.5%; P = 0.52) or SRNS (15.4%; P = 0.0056). one SSNS case, three FRNS/SDNS, and three SRNS carried the *NPHS1* R1109X (Fin-minor) variant. For *NPHS2*, one SSNS case carried a homozygote R168H variant, and another SSNS case carried a compound heterozygote R229Q variant. The allele frequencies of the *NPHS1* R1109X variant and the *NPHS2* R168H and R229Q variants were 14% and 4.3%, respectively. No cases carried the *NPHS1* L41NfsX50 variant. Available kidney biopsy reports revealed that minimal change disease was most frequent in FRNS/SDNS cases (56.7%) and focal segmental glomerulosclerosis, most common in SRNS cases (75%).

Conclusion: A significant proportion of the youngest SSNS cases may develop FRNS/SDNS.

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Keywords: Nephrotic syndrome; NPHS1; NPHS2; Nephrin; Podocin; Frequently-relapsing or Steroid-dependent; Steroid-resistance

Abbreviations: CRF -chronic kidney failure; FATHMM-Functional Analysis through Hidden Markov Models; FRNS -Frequently Relapsing Nephrotic Syndrome; FSGS- Focal Segmental Glomerulosclerosis; GFR- Glomerular Filtration Rate; IgA- Immunoglobulin A; ISKDC-International Study of Kidney Disease in Children; Lo Ftool- loss-of-Function Tool; MCD-Minimal Change Disease; MCNS-Minimal change Nephrotic Syndrome; MsPGN-Mesangioproliferative glomerulonephritis; NPHS1-Nephrin; NPHS2-podocin; NS- Nephrotic Syndrome; PolyPhen-2- Polymorphism Phenotyping v2, SA- Saudi Arabia SDNS- Steroid-dependent Nephrotic Syndrome; SIFT- Sorting Intolerant from Tolerant, SRNS -steroid-Resistant Nephrotic Syndrome; SSNS- Steroid-Sensitive Nephrotic Syndrome

Introduction

Nephrotic syndrome (NS) is the most common diagnosis in pediatric nephrology and is evolving as a leading cause of uremia [1]. Affected individuals have massive proteinuria *in utero*, and NS develops soon after birth (especially in premature infants) [2]. Associated with proteinuria, other visible signs that appear soon after birth include hypoalbuminemia, hyperlipidemia, edema, and abdominal distention [2]. Electron microscopy of the kidneys in patients with NS has shown blurring of the podocytes and the absence of split diaphragms. Early bilateral nephrectomy followed by kidney transplantation in infants is the only curative therapy for NS, and the disorder is otherwise lethal [3]. Thus, *NS* diagnoses are based on the clinical features of the affected neonate and kidney histopathology. NS can be suspected prenatally based on increased α-fetoprotein in the maternal serum or amniotic fluid [4].

For decades, NS has been categorized, based on its clinical response to steroid medications, as either steroid-sensitive NS (SSNS) or steroid-resistant NS (SRNS) [5]. About 80% of NS cases are steroid sensitive. Of these individuals with SSNS, 70% will suffer from frequent relapses, and a significant proportion may develop frequently relapsing NS or steroid-dependent NS (FRNS/SDNS) [1]. Children with FRNS/SDNS are often treated with repeated corticosteroid courses [6], as a second-line treatment, with non-corticosteroid immunosuppressive medications, often associated with heavy treatment burden [7]. In addition, 20% of sporadic children with SRNS may face a therapeutic obstacle to pediatricians and nephrologists. Kidney histology revealed focal segmental glomerulosclerosis (FSGS), minimal change nephrotic syndrome (MCNS), and mesangioproliferative glomerulonephritis (MsPGN) in 75%, 20% and 5% of the SRNS patients, respectively [8-10]. In about 14% of patients with FSGS [11,12], deformities in the podocytes and split diaphragm can be explained by genes such as *INF2* (MIM 610982), *ACTN4* (MIM604638), *TRPC6* (MIM 603652), *WT1* (MIM 607102), *NPHS1* (MIM 602716), and *NPHS2* (MIM 604766) [11]. Treatment with cyclosporin A is much more effective in patients with nonhereditary SRNS than in those with genetic-based SRNS [12]. suggesting that genetic variation contributes to the pathogenesis of FSGS and influences the outcome of clinical treatment. Thus, it is of great clinical significance to elucidate the genetic characteristics of FSGS.

Variants in the *NPHS1* gene have been identified as a cause of congenital NS of the Finnish type [13], and *NPHS1* is one of the most common genes associated with SSNS. *NPHS1* is located at chromosome 19q13.1 and contains 29 exons spanning 150 kb. Nephrin, the transmembrane protein encoded by *NPHS1*, is produced only in glomerular podocytes, where it participates in the intercellular junctions of mature podocytes and slit diaphragm formation [14]. Nephrin also serves as the main component of the glomerular filtration barrier [15]. Reduced expression of nephrin could result in progressive proteinuria with glomerular hypertrophy and FSGS [16]. The prevalence of *NPHS1-associated NS* has increased worldwide, although more than half of the cases were diagnosed in Finland, where the prevalence is 1 in 8,200 births [17]. Despite its association with congenital NS of the Finnish type, *NPHS1* has been confirmed as a susceptibility gene for various kidney diseases, including SRNS, FSGS, MCD, and IgA nephropathy [14,18-21].

Among Finnish children with congenital NS of genetic origin, nearly 78% have the p.Leu41fsX90 variant in exon 2 of *NPHS1*, and nearly 16% have the p.R1109X variant in exon 26 of *NPHS1* [13]; these variants, which are referred to as Fin-major and Fin-minor, respectively [22,23], have rarely been reported in non-Finnish ethnic populations [14]. *NPHS1* genetic screening of non-Finnish patients has shown that the frequency of *NPHS1* variants is lower than that in Finnish patients (39% versus 55% of NS cases) [18,20].

Most patients with idiopathic NS respond to steroid therapy and show a favorable outcome, but 20% are resistant to steroid therapy, with progression to end-stage kidney failure in many cases. A subgroup of nephrosis referred to as steroid-resistant idiopathic NS has been investigated [22] and is characterized by familial occurrence, age of onset in early childhood, resistance to steroid therapy, progression to end-stage kidney disease within a few years, and absence of recurrence after kidney transplantation.

NPHS2 is a gene encoding podocin, another important protein located at the split diaphragm. *Podocin* is an integral protein

consisting of 383 amino acids having a single short hairpin-like transmembrane domain and cytosolic N- and C-terminal domains [24]. It is expressed in the podocyte-foot process cell membrane at the split diaphragm's insertion site and might maintain the integrity of the split diaphragm [25]. Pathogenic variants in *NPHS2* can result in a reduction or absence of functional proteins and thus impair the formation of normal slit diaphragms [26,27]. This abnormality leads to SRNS before six years of age, and patients reach end-stage kidney disease during their first decade of life [26]. Several reports have demonstrated that variants in the *NPHS2* gene represent a frequent cause of SRNS, occurring in 20-30% of sporadic SRNS cases [27,28], *NPHS2* gene is frequently associated with FSGS, where their variants in *NPHS2* can cause autosomal recessive SRNS. Several genetic variants of the *NPHS2* gene, including R229Q and R291W, have been described in patients with NS, and individuals carrying both variants have shown progressive loss of kidney function. The p.R229Q variant (c.686G>A; rs61747728), a nonsynonymous variant in exon 5, is affirmed to be one of the most important predictive factors in the pathogenesis of SRNS [29-31]. Evidence has confirmed that the R229Q allele is a disease-causing variant rather than a benign polymorphism [29]. However, susceptibility in individuals with R229Q variants varies among ethnic groups. A wide range of incidence in pediatric idiopathic NS syndrome worldwide is reported to be 1.15-11.6 per 100,000 children [32], whereas the risk of average annual incidence has been reported higher in Asian (7.14/100,000) and Black (3.53/100,000) children compared to White Caucasian (1.83/100,000), and or American (2.13/100,000) children.

Given the *NPHS1* and *NPHS2* variants commonly reported worldwide in recent decades, the present study analyzed these variants in Saudi children with the early-onset presentation of NS and identified the types of NS and phenotypes associated with the different variants.

Subjects and Methods

Ethics statement and consent

Molecular analyses were performed at the DNA Diagnostic Laboratories, Medical Genetics Department, College of Medicine, Umm Al-Qura University. This study was conducted under the Declaration of Helsinki. The Institutional Biomedical Ethics Committee-Umm Al-Qura University (reference #HAPO-02-K-012), licensed from the National Committee of Medical and Bioethics (King Abdelaziz City for Science and Technology-Riyadh), approved the study. The parents or guardians of 35 children with NS and 40 healthy controls gave informed consent before participating in the study.

Definition and management

We followed the protocol of the International Study of Kidney Disease in Children (ISKDC) [33] ,which categorized NS cases based on their response to treatment: 1) Uncomplicated SSNS: relapse ≤ 4 times/year and have not required treatment with steroid-sparing agents. 2) SRNS: no response to steroid treatment after four weeks of enteral prednisone at 60 mg/m^2 per day in addition to three intravenous administrations of methylprednisolone at 1,000 mg/1.73 m^2 per dose. 3) FRNS/SDNS: relapse ≥ 2/month (FRNS) or have had two consecutive relapses during the tapering period or relapsed within two weeks of discontinuing steroid therapy (SDNS).

The Schwartz formula was used to estimate the glomerular filtration rate (GFR) in NS cases. However, GFR = k * height (cm)/ plasma creatinine (mg/dL): k is 0.45 for infants (age ≤ 1.5 years), 0.55 for older children and adolescent girls, and 0.70 for adolescent boys > 13 years of age. Decreased kidney function was defined as GFR ≤ 90 ml/min/1.73m^2, chronic kidney failure (CRF) as GFR ≤ 60 ml/min/1.73m^2, and end-stage kidney disease as GFR ≤ 15 ml/ min/1.73m29 [5].

Study population

A total of 35 children with histologically proven NS were included in this study. Cases were diagnosed in pediatric nephrology clinics of different hospitals by nephrologists in Western regions of Saudi Arabia, including Makkah, Al-Hada, and Al-Taif governorates. The diagnosis of NS was established based on histological examination of a specimen of kidney tissue gained by kidney biopsy. The clinical diagnosis of NS required the presence of heavy proteinuria (>50 mg/kg/day or urine protein/creatinine ratio ≥ 2 mg/mg) and hypoalbuminemia (<2.5 mg/dl). Clinical records were gathered, including family history, age at onset, gender, frequency of relapse, kidney biopsy reports, GFR, and treatment modes. Cases with postinfectious glomerulonephritis and systemic diseases (e.g., lupus erythematosus, diabetes, amyloidosis, vasculitis, metabolic or toxic nephritis, hepatitis B, or hereditary glomerular disease) were excluded from this study. Controls included 40 children with no personal or family history of kidney disease (Figure 1).

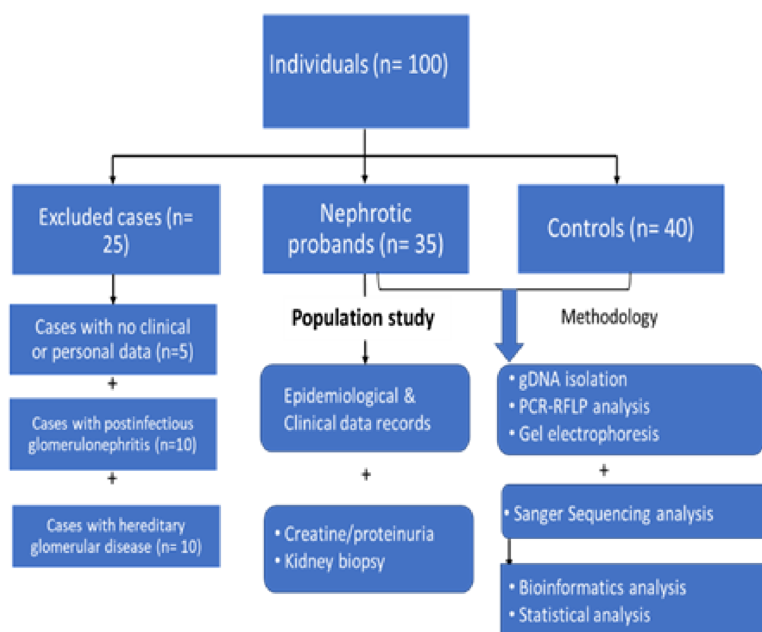

Figure 1: Participant eligibility and applied methodology.

DNA analysis

Genomic DNA samples were isolated from buccal cells by gently scraping the mucosa using an Oragene DNA-OGR-575 kit (DNA Genotek Inc, Ottawa-ON, Canada) following some modifications [34]. Sometimes, DNA was isolated from peripheral blood samples using a DNA extraction Spin Column kit (Qiagen, USA).

PCR Conditions

Genomic DNA was subjected to a 25-µl reaction volume of 0.5 mM of each primer, 200 µM of each dNTP, 67 mM Tris-HCl, 16 mM $(NH_4)_2SO_4$, 0.01% Tween-20, 1 mM $MgCl_2$, and 0.15 units of *Taq* DNA polymerase. The samples were then subjected to DNA denaturation at 95˚C for 5 min, followed by 30 cycles of denaturing for 1 min at 95˚C, annealing at 58˚C for 30 s, and extension for 30 s at 72˚C, with a final extension of 7 min at 72˚C in a PCR Engine Dyad thermocycler (Bio-Rad Laboratories Inc, Hercules, CA). The primers were designed based on previously published sequences for the *NPHS1* gene (L41NfsX50 and R1109X loci) and the *NPHS2* gene (R229Q, and R291W loci) [13,35]. Oligonucleotide primer sequences are listed in [Table 1].

Table 1: Amplification conditions including oligonucleotide sequences, enzymatic temperature, and fragment sizes.

Gene	Variant	Exon	Oligonucleotide Sequences (5`-3`)	Restriction Enzyme	Digest Temperature	Fragment Size (bp)
NPHS1	L41NfsX50	2	F: TTCTGGGCCCTGCCTGAAATC	TaqI	65ºC (1 hr)	103 (83+20)
			R: CATCTTTGGCCCATTGCAC			
NPHS1	R1109X	26	F: GGGGCTTGCATAGGGTCACT	AvaII	37ºC (2 hr)	239 (188+51)
			R: AGTGTCCCGCTCTCCTGTCC			
NPHS2	R229Q	5	F: AGGATTTACCACAGGATTAAGTTGTGCA	ClaI	37ºC (2 hr)	545 (364+181)
			R: TAGCTATGAGCTCCCAAAGGGATGG			
NPHS2	R291W	7	F: AGGCTTGCAAGTCTGTGTGAAAGC	PflMI	37ºC (2 hr)	283 (155+128)
			R: AGGAAGCAAAGGGGAAATGTTCTCC			

Enzymatic digestion of *NPHS1* and *NPHS2* Loci

We identified the *NPHS1* (L41Nfs50 and R1109X) and *NPHS2* (R229Q and R291W) variants using enzymatic digestion of their PCR fragments. Briefly, the PCR amplicons of these variants were incubated with *Taq*I, *Ava*II, *Cla*I, and *Pfl*MI endonucleases for 2 hours according to the manufacturer's instructions (New England Biolabs Inc., MA, USA) (Table 1). The digested fragments were separated on a 3%MetaPhor agarose gel (Lonza Rockland, Inc., USA) for 2 hours at 3.5 volts/cm. The digested fragments were visualized under a UV transilluminator and photographed using a Gel Documentation System (UVitec, Cambridge, UK). Genotyping results were confirmed by sequencing 20% of the samples (Figure 2a,b).

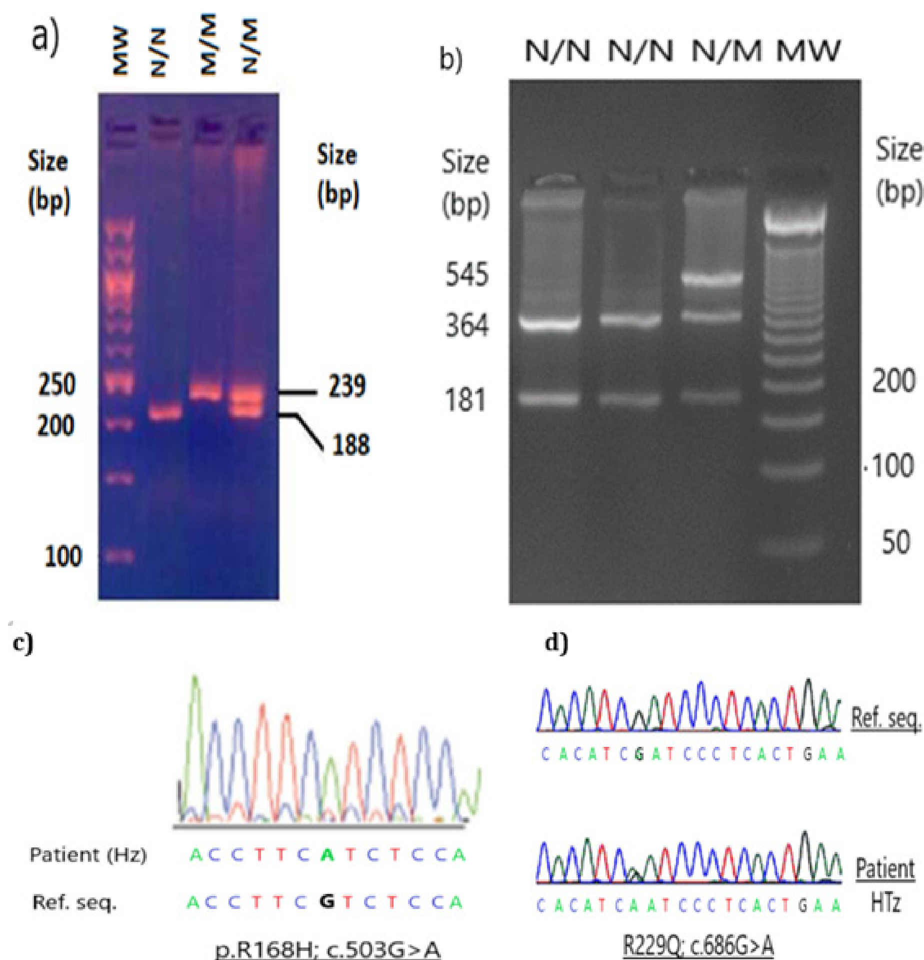

Figure 2: Molecular analyses of *NPHS1*, and *NPHS2*.

a) Electrophoretic gel pattern identified the *NPHS1* (R1109X) using AvaII restriction enzyme.

b) The uncut PCR product (239-bp band, lane M/M) shows a homozygous R1109X variant. Heterozygosity (N/M) of the R1109X variant is detected by three fragments (239-, 188-, and 51-bp). The 51-bp fragment is out of the gel. b) As for the *NPHS2* (R229Q) variant, the gel pattern showed the cutting of the amplified DNA fragment with the *ClaI* restriction enzyme. Lane N/M represents the compound heterozygosity of case #19 (364- and 181-bp bands). The R229Q allele is shown by the 545-bp band.

c) DNA sequence analysis of NPHS2 in case #36 showed a homozygous R168H (c.503G>A) variant in exon 4.

d) Direct Sanger sequencing of the PCR fragments of the *NPHS2* gene identified a heterozygous variant (p.R229Q; c.686G>A, exon 5) from case #17. The chromatogram of the patient is aligned with the reference sequence (SeqA 6.Ink software, ABI, Thermo Fisher Scientific, USA).

Sequence analysis of the whole *NPHS2* Gene

For mutation analysis, Sanger sequencing was carried out using oligonucleotide primers optimized for all exonic regions of *NPHS2*. Briefly, PCR amplicons were purified using SPRI paramagnetic bead-based chemistry to remove contaminants, including dNTPs, salts, primers, and primer dimers, according to the manufacturer's instructions (AMPure XP, Beckman Coulter Life Sciences, IN, USA). The purified products were treated with the Big Dye Terminator v3.1 cycle sequencing kit and then sequenced using the 3500 Genetic Analyzer sequencer system (Thermo Fisher Scientific, USA). Oligonucleotide sequences of eight exons, including flanking regions, were previously reported (Table S1). The full oligonucleotide primers optimized for *NPHS2* gene can be found in the Supplement (see supplementary file). Variants were reviewed and aligned using dbSNP (Sequence Analysis Software ver. 6, ABI, Thermo Fisher, USA) (Figure 2c,d).

Bioinformatics analysis

For nonsynonymous variants, we used the *in silico* tools Sorting Intolerant from Tolerant (SIFT), Polymorphism Phenotyping v2 (PolyPhen2), MutationTaster, Functional Analysis through Hidden Markov Models (FATHMM), Mutation Assessor, and loss-of-function tool (LoFtool) to predict the effects of the variants on their corresponding functional proteins (https://www.ensembl.org/vep/) (Table 2).

Table S1: Oligonucleotide primers optimized for *NPHS2* gene.

Exon	Forward (5`-3`)	Reverse (5`-3`)
exon 1	GCAGCGACTCCACAGGGACT	TCCACCTTATCTGACGCCCC
exon 2	AGAATTGGACCAACAGATGC	AAGTGAGAATGGGCATGGTG
exon 3	GCCCCCGCCGTCTTATGCCAAGGCCTTTTGAAGAC	GGGTTGAAGAAATTGGCAAGTCAGG
exon 4	AAGGTGAAACCCAAACAGC	CGGTAGGTAGACCATGGAAA
exon 5	AGGATTTACCACAGGATTAAGTTGTGCA	TAGCTATGAGCTCCCAAAGGGATGG
exon 6	TATTATAAATAAGGCACTGTGAAGTTAAATACAAC	CCCCCGCCCGCCAGAATATTTTCCTTTATCATACAG
exon 7	GAGGCTTGCAAGTCTGTGTGAAAGC	AGGAAGCAAAGGGGAAATGTTCTCC
exon 8	CGTCCCGCGTGAAGCCTTCAGGGAATGAAGAAC	GCGTCCCGTTCTATGGCAGGCCCCTTTACAGTC

Table 2: *In silico* functional predictions of non-synonymous *NPHS1* and *NPHS2* variants detected in this study.

Gene	SNP ID	A.A. Change (Codons)	cDNA/ CDS (A.A. Position)	MAF	SIFT	Poly Phen2	Mutation Taster	FATHMM	PROVEAN	Mutation Assessor	LoF Tool	Clinical Significance
NPHS1	rs386833873	L/X CTG/G	788-789/ 121-122 -41	0.0011	NA	NA	Disease causing	NA	NA	NA	0.574 Possibly damaging	Pathogenic
NPHS1	rs137853042	R/* CGA/TGA	3992/3325 -1109	0.0002	NA	NA	Disease causing	NA	NA	NA	0.574 Possibly damaging	Pathogenic
NPHS2	rs530318579	R/H CGT/CAT	587/503 -168	0.0002	0.0 Deleterious	0.999b Probably damaging	Disease causing	−3.51 Damaging	−4.8 Damaging	3.735 High	0.251 Damaging	Pathogenic/ likely pathogenic
NPHS2	rs61747728	c.686G>A CGA>CCA	770/686 -229	0.015	0.14 Tolerate	0.313 Benign	Disease causing	−3.57 Damaging	−2.1 Neutral	1.68 Low	0.251 Damaging	Pathogenetic‡

*Pathogenic, uncertain-significance, conflicting-interpretations of pathogenicity, risk-factor.

CDS, Coding Sequence; FATHMM, Functional Analysis Through Hidden Markov Models; LoFTool, Loss-of-function tool (https://www.ensembl.org/vep); MAF; minor allele frequency; PolyPhen2, Polymorphism Phenotyping-2; PROVEAN; SIFT, Sorting Intolerant from Tolerant.

NPHS1-NPHS2 gene interaction

The Search Tool for Retrieval of Interacting Genes (STRING) database (version, 11.5) (**https://string-db.org**) was also used to predict functional interactions between proteins.

Statistical analysis

The paired *t*-test and one-way ANOVA with *chi*-square (χ^2) values were applied to evaluate the demographic and clinical characteristics of NS cases using MedCalc (https://www.medcalc.org) and Social Science Statistics (https://www.socscistatistics.com/tests/chisquare2/default2.aspx) software.

Results

Among the 35 cases with NS, the female-to-male ratio was 13: 22 (i.e., 1:1.7), with no significant difference between genders (t-value = 0.329, 95% Cl, 4.1-6.3; P = 0.745). Additionally, no significant differences ($P > 0.05$) were found between cases and controls regarding age or gender. Among our cases, the mean age of onset was 3.0 ± 2.13 years (range, stillbirth-8 years). Six cases (17.1%) had an age of onset between stillbirth and 12 months, 21 cases (60%) had an age of onset >1-5 years, and 8 (22.9%) had an age of onset >5-8 years. Cases with FRNS/SDNS (46.2%) had a significantly younger age at presentation than cases with SSNS (38.5%) or SRNS (15.4%) (Figure 3).

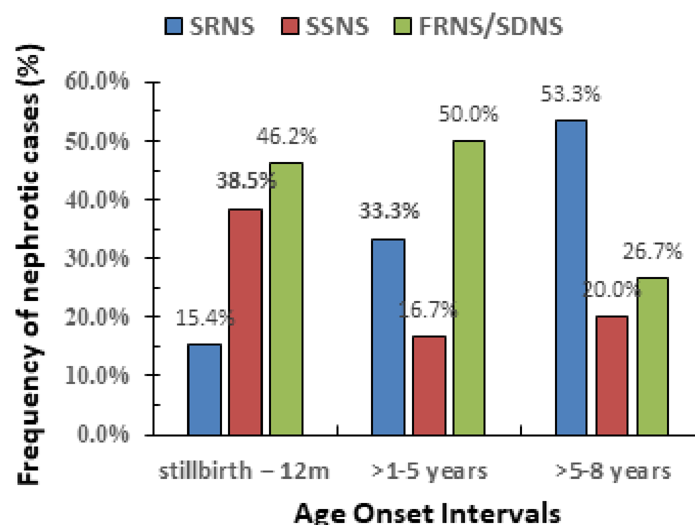

Figure 3: Prevalence of cases with nephrotic syndrome and therapeutic steroid response by age of onset.

*NS, nephrotic syndrome; SRNS, steroid-resistant nephrotic syndrome; SSNS, steroid-sensitive nephrotic syndrome; FRNS/SDNS, frequently relapsing/steroid-dependent nephrotic syndrome.

Allelic variants of *NPHS1* and *NPHS2*

Our study found no cases due to the frameshift L41fsNX50 (c.121_122delCT) variant within the *NPHS1* gene. Moreover, ten alleles leading to nonsense R1109X (c.3325C>T) variants were observed in exon 26 (Table 3). R1109X variants were associated with three homozygous NS cases (cases #2, #3, and #4) and four heterozygous NS cases (#11, #13, #16, and #24). Thus, the allele frequency of the R1109X variant in *NPHS1* reached 14% (10/70 alleles). As for *NPHS2*, none of the cases with NS had the R291W (c.871C>T) variant. However, we detected the missense R168H (c.503G>A) variant in exon 4 and the R229Q (c.686G>A) variant in exon 5. The allele frequency of these *NPHS2* variants was 4.3% (3/70 alleles).

Table 3: Clinical data of nephrotic cases with the *NPHS1* and *NPHS2* variants.

Case#	Gender	Age of Onset	FH (Consanguinity)	Steroid Response	Kidney Biopsy Result	Serum Creatinine (µmol/L)	GFR ml/ min/ 1.73 m2	Medications	Variant (Genotype)
2	M	2.0 y	No (no)	FRNS/SDNS	MCD	44	102.8	Steroid, CsA, MPD	R1109X (Hz)
3	M	18 m	No (no)	SRNS	FSGS	22	138.6	Steroid, FK506	R1109X (Hz)
4	M	4.5 y	No (no)	SSNS	FSGS	14	158	Steroid	R1109X (Hz)
11	F	5.0 y	No (no)	FRNS/SDNS	MCD	28	153	Steroid, Cytoxan, FK506	R1109X (Ht)
13	F	6.0 y	No (no)	SRNS	FSGS	NA	NA	Steroid, Cytoxan, CsA, MMF	R1109X (Ht)
16	F	5.0 y	No (no)	FRNS/SDNS	MCD	26	199	Steroid, Levamisole	R1109X (Ht)
17	F	17 m	Yes (no)	SSNS	MCD	30	108	Steroid, CsA	R229Q (Ht)
24	F	9.0 y	No (yes)	SRNS	FSGS	43	134	Steroid, Cytoxan, FK506, MMF	R1109X (Ht)
36	M	9.0 y	No (no)	SSNS	MCD	38	163.3	Steroid, Cytoxan, FK506, MMF	R168H (Hz)

*CsA, Cyclophosphamide A; F, female; FH, family history; FRNS/SDNS, frequently relapsing/steroid-dependent nephrotic syndrome; FSGS, focal segmental glomerulosclerosis; Ht, heterozygote; Hz, homozygous; M, male; MCD, minimal change disease; NA, not available; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SRNS, steroid-resistant nephrotic syndrome.

Response to steroid therapy

Among the NS cases, 12 (34.3%) were SRNS, 8 (22.9%) were SSNS, and 15 (42.9%) were FRNS/SDNS. (Figure 3) presents the age of onset of the cases and their responses to standard steroid therapy. Cases with FRNS/SDNS were significantly more likely to have an early age of onset stillbirth-12 months, or >1-5 years (46.2% or 50.0%, respectively) than were those with SSNS (38.5% or 16.7%) or SRNS (15.4% or 33.3%). In contrast, SRNS cases were more likely to have a later age of onset (>5-8 years) than were SSNS or FRNS/SDNS cases (53.3% vs. 20.0% vs. 26.7%, respectively). The response to steroid therapy in SRNS cases versus SSNS+FRNS/SDNS cases showed a χ^2 = 4.1 (P = 0.044) for age of onset stillbirth-12 months, χ^2 = 9.3 (P = 0.0023) for age of onset >1-5 years, and χ^2 = 0.19 (P = 0.663) for >5-8 years.

Kidney biopsy outcome

Kidney biopsy results were reported for 30 (85.7%) of 35 cases: 17 cases (56.7%) with MCD, 12 cases (40%) with FSGS, and 1 case (3.3%) with membranoproliferative glomerulonephritis (MPGN). MCD was most frequently reported in FRNS/SDNS cases (17/30 cases, 56.7%), whereas FSGS was the predominant histological phenotype in SRNS cases (9/12 cases, 75%) (Figure 4).

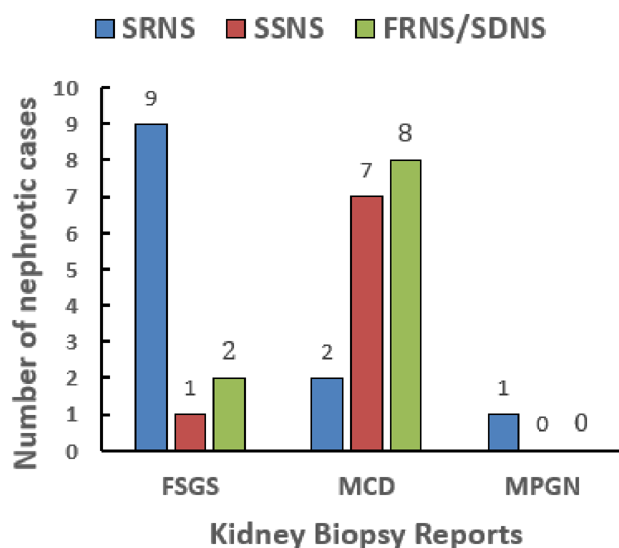

Figure 4: Kidney biopsy findings among the clinical groups (n= 30).

*NS, nephrotic syndrome; SRNS, steroid-resistant nephrotic syndrome; SSNS, steroid-sensitive nephrotic syndrome; FRNS/SDNS, frequently relapsing/steroid-dependent nephrotic syndrome; FSGS, focal segmental glomerulosclerosis; MCD, minimal change disease; MPGN, membranoproliferative glomerulonephritis.

Genotype-phenotype correlation

(Table 3) represents the phenotype-genotype correlations between NS phenotypes and *NPHS1/NPHS2* variants. Among cases with *NPHS1 variants*, three (cases #3, #13, and #24) had SRNS, three (cases #2, #11, and #16) had FRNS/SDNS, and one (case #4) had uncomplicated SSNS. Clinical and kidney biopsy analyses were used to diagnose four cases with FSGS and three cases with MCD.

Among the cases with *NPHS2* variants, two cases were SSNS. Case #36, who was homozygous for the R168H variant, was a boy with no history of kidney disease. At nine years old, he was diagnosed with MCD using kidney biopsy analysis. His serum creatinine concentration was 38 µmol/L (normal range, 35-53 µmol/L), and his GFR was 163 ml/min/1.73 m^2. There was no family history, and the parents were not consanguineous. His condition was treated with standard steroid medications: cyclophosphamide, FK506®, and MMF®, and described as a steroid-sensitive response (Table 3). Another patient carrying the *NPHS2* R229Q variant (case #17) was a 17-month-old girl with a positive family history of proteinuria and kidney disease with no consanguinity of parents. A kidney biopsy diagnosed her as having MCD. Her condition was steroid-dependent and treated with cyclosporine A. Her serum creatinine was 30 µmol/L at onset, and her GFR was 108 ml/min/1.73 m^2.

NPHS1-NPHS2 Protein interactions

The protein-protein interaction (PPI) network of the *NPHS1-NPHS2* protein-coding gene loci created by STRING software (https://string-db.org/) showed significantly more interactions than would be expected for a random set of proteins of the same size and degree of distribution drawn from the genome (PPI enrichment *P*-value = 0.00895) (Figure 5). More extended PPI network of the *NPHS1-NPHS2* gene loci, including *FYN, NCK1, TRPC6, CD2AP,* and *WASL* protein-coding gene loci, were strong significant associations (PPI enrichment *P*-value = 2.64x10^{-5}) (Table 4).

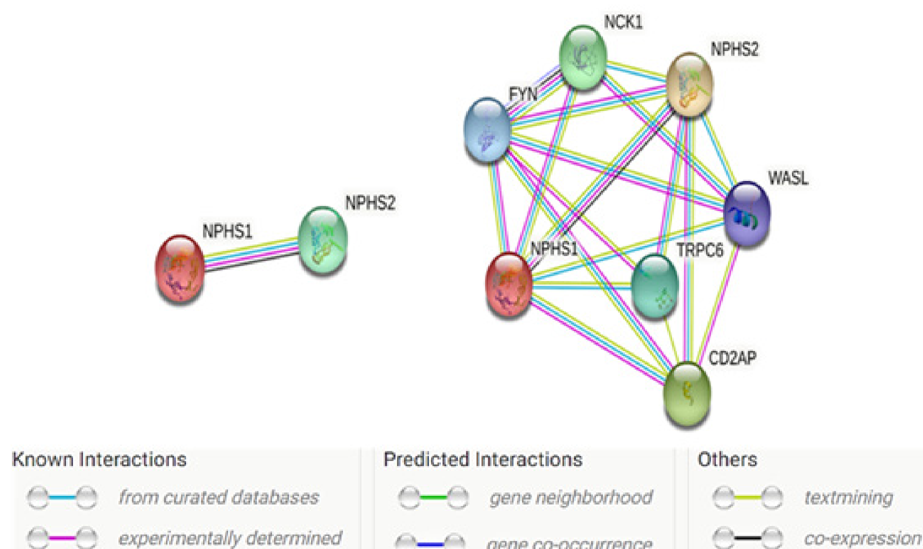

Figure 5: Protein-protein interaction (PPI) network presented the *NPHS1*/*NPHS2* gene cluster created by STRING (https://string-db.org/).

Left side: Strong significant interactions between the *NPHS1* and *NPHS2* protein-coding gene loci.

Right side: More extended PPI network includes seven *NPHS1, NPHS2, FYN, NCK1, TRPC6, CD2AP*, and *WASL* genes (average node degree = 5.14) with 18 edges are shown.

*N.B. Each node represents all the proteins produced by a single, protein-coding gene locus. Colored nodes describe proteins and the first shell of interactors. Edges represent protein-protein associations meant to be specific and meaningful, i.e., proteins jointly contribute to a shared function; this does not necessarily mean they are physically binding each other.

Table 4: Functional enrichment in NPHS1-NPHS2 protein-coding gene loci Network.

Gene Ontology (GO) term	Description	Count in Network	Strength	FDR
Biological Process (GO):				
GO:0072015	Glomerular visceral epithelial cell development	2/11	3.25	0.0052
GO:0007588	Excretion	2/44	2.65	0.0087
Cellular Component (GO):				
GO:0036057	Slit diaphragm	2/9	3.34	0.00049
Local network Cluster (STRING):				
CL:16389	Glomerulosclerosis	2/5	3.59	0.0005
Reactome Pathways:				
HSA-373753	Nephrin family interactions	2/23	2.93	0.0034
Disease-Gene Associations (DISEASES):				
DOID:10966	Lipoid nephrosis	2/3	3.81	0.00023
DOID:1312	FSGS	2/10	3.29	0.00075
DOID:2590	Familial NS	2/11	3.25	0.00075
Tissue Expression (TISSUES):				
BTO:0004631	Glumerular endothelium	2/2	3.99	0.00006
BTO:0002295	Podocyte	2/6	3.51	0.00014
Subcellular Localization (COMPARTMENTS):				
GOCC:0098846	Podocyte foot	2/4	3.69	0.00018
GOCC:0036057	Slit diaphragm	2/10	3.29	0.0004
GOCC:0036056	Filtration diaphragm	2/10	3.29	0.0004

*NS, nephrotic syndrome; FSGS, focal segmental glomerulosclerosis; FDR, False discovery rate

Discussion

In the present study, we explored variants in the *NPHS1* (nephrin) and *NPHS2* (podocin) genes and their association with disrupting the slit diaphragm architecture in the pathogenesis of earlier-onset NS. One known nonsense variant (R1109X) in *NPHS1* and two missense variants (R168H and R229Q) in *NPHS2* were reported here. Based on kidney biopsy reports, the identified variants were associated with MCD, FSGS, and MPGN phenotypes.

Based on electron microscopy analysis, abnormalities of the slit diaphragm have been seen in NS cases with MCD as the most common form of SSNS [36]. In addition, Horinouchi, et al. [37], have reported reduced podocin expression in patients with FSGS but not in those with MCD, suggesting that podocin expression and function in the slit diaphragm may not be altered in MCD.

Although *NPHS1* variants were first reported in congenital NS of the Finnish population, subsequent studies have confirmed that *NPHS1* is also a causative gene or susceptibility gene for kidney diseases such as FSGS, MCD with NS, IgA nephropathy, and MPGN [14,18-21,38]. The L41Nfs50 and R1109X variants in *NPHS1* occur in the Finnish population at 78% and 16%, respectively[13], but rarely in non-Finnish ethnic populations[14]. Interestingly, 14% of NS cases carried the R1109X (Fin-minor) variant in our sample, but none carried the L41NfsX50 (Fin-major) variant. About 150 *NPHS1* variants have been described among NS patients in different non-Finnish ethnic populations, including small insertions and deletions (indels), nonsense variants, and splicing variants [20,23,38-42]. Mostly, missense variants result in retention of nephrin in the endoplasmic reticulum, leading to complete loss of nephrin from the cell surface [43]. In addition, some reports have described silent variants that cause specific phenotypes by influencing mRNA structure or inactivating genes that affect gene splicing machinery and lead to exon skipping [44].

In Chinese cases with sporadic SRNS, synonymous changes in the *NPHS2* gene (e.g., T741T, V763V, S1105S, A318A, and L346L) were observed in cases and healthy individuals, although there were no significant differences between cases and controls for some of these changes [44,45]. Large cohorts with familial SRNS in Europeans and North Americans have shown detection rates of 38% and 26% for homozygous and compound heterozygous *NPHS2* variants, respectively, but 6-19% for *NPHS2* variants in sporadic NS cases [8,28]. Two studies have confirmed that cases with FSGS and two pathogenic *NPHS2* variants are generally characterized by early-onset disease, response to SRNS treatment, and reduced risk of FSGS recurrence after kidney transplantation [28]. The R229Q and R291W variants in *NPHS2* were previously reported as compound heterozygotes [46,47], detected in 22 (84.6%) of 26 SRNS cases, 42.3% of whom went on to develop end-stage kidney disease (ESKD) [28].

Although only one compound heterozygous R229Q variant (1/70; 1.4%) was detected in the present study, Mikó, et al. [48]. Described this variant as the most frequent *NPHS2* variant in the general population and as the first human variant for which pathogenicity depends on another associated allele. The R229Q variant can cause FSGS if it is *trans*-associated with specific variants that affect the protein region spanning 270-351 residues [48]. The coexistence of the R229Q variant and other variants in residues 260 and 310 has been reported in three children who developed ESKD [49]. More recently, Righetti, et al. [50]. concluded that compound heterozygous R229Q or R291W variants might be associated with the FSGS phenotype, but neither heterozygous variant was associated with significant proteinuria.

Conclusion

Our study demonstrated that the nonsense R1109X variant of the Finnish type in the *NPHS1* gene is present in NS patients with sporadic FSGS and MCD in the Saudi community. *NPHS1* variants could represent an important role in the pathogenesis of these diseases, but more research is needed to explain the physiopathological mechanisms of these variants. Moreover, nearly 50% of Saudi cases at younger ages with FRNS/SDNS were still challenging during repeated protocols of corticosteroids and second-line immunosuppressive agents associated with morbidity. Although the allele frequency of the *NPHS2* gene in cases with NS was 4.3% (3 of 70 cases) in our study, the sample size was relatively small. During the preparation of this manuscript, external exome sequencing for 73 of our NS cases revealed two additional previously unreported variants that are yet to be confirmed in *NPHS1* and *NPHS2*. Thus, extending our research studies using exome sequencing could help expedite the discovery of NS genetic heterogeneity among the Saudi community.

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Conflict of Interest

The authors report no conflicts of interest in this work.

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