Nephrotic syndrome in childhood: genotype-phenotype association studies and screening for novel mutations

Catarina Sofia Urbano Silveira

Dissertação
Mestrado em Biologia Molecular e Genética

Versão Pública

2013
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Dissertação orientada pelo Doutor Gabriel Miltenberger-Miltenyi e pelo Professor Francisco Dionísio

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ACKNOWLEDGMENTS

This project was supported and is a part of an investigation project of the Portuguese Society of Nephrology.

I would like to thank to the persons who were behind the main project and made this possible:

Dr. Gabriel Miltenberger-Miltenyi, who is the principal responsible for this thesis, thanks for the time, patience and precious help.

To Dr.ª Ana Rita Sandes, Dr.ª Leonor Real Mendes and Dr.ª Margarida Almeida from the Pediatric Nephrology Department of Hospital de Santa Maria and Prof. Dr. Fernando Coelho Rosa from the Instituto de Ciências da Saúde da Universidade Católica Portuguesa, thank you for relying on my hands the practical part of this project and, by allowing the use these results, make this thesis possible.

I would like of course to thank to the children, and their families, that were part of this study.

Then, and without any order of importance, I would like to thank to those who, even without realizing, were there to support me:

I would like to thank to Dr.ª Teresa Porta Nova, technical director of GenoMed, who allowed the development of this project at the lab and who always gave me support to carry on.

I would also like to thank to Prof. Francisco Dionísio who promptly accepted to be my supervisor and was there to help me with all the questions related with the master thesis.

Thanks to Sónia Pereira, that did a part of the bench work because some of these cases were done and analyzed by her even before I arrived to GenoMed.

Thanks to André Janeiro and Sara Malveiro who were always available to check the structure, read, correct and give their opinions on what I was writing, even if they had to lose one or two Sundays to help me with this.
To Prof. Francisco Enguita, thank you so much for the time, support and precious help.

To Professora Dr.ª Fernanda Carvalho, I would like to thank the help and the time that she spent to explaining me the histological part of nephrotic syndrome.

I would like to thank to my family, especially my parents, and to my friends for the support and patience during this period, which was not always easy. I promise that I will spend more time with you now.

A big thanks to a special person, my appendix, for the support, patience, dedication and for not letting me give up even when the discouragement seemed to be the leader of my actions. Thank you for being by my side.

Without you all this work would not be possible, thank you so much.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AA</td>
<td>Amino Acid</td>
</tr>
<tr>
<td>Abs</td>
<td>Absorbance</td>
</tr>
<tr>
<td>AD</td>
<td>Autosomal Dominant</td>
</tr>
<tr>
<td>AR</td>
<td>Autosomal Recessive</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>CNF</td>
<td>Congenital nephrotic syndrome of the Finnish type</td>
</tr>
<tr>
<td>CNS</td>
<td>Congenital Nephrotic Syndrome</td>
</tr>
<tr>
<td>ddNTPs</td>
<td>Dideoxynucleotides</td>
</tr>
<tr>
<td>DMS</td>
<td>Diffuse Mesangial Sclerosis</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Deoxynucleotide</td>
</tr>
<tr>
<td>e.g.</td>
<td>example given</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage Renal Disease (Stages 4 and 5)</td>
</tr>
<tr>
<td>FSGS</td>
<td>Focal and Segmental Glomerulosclerosis</td>
</tr>
<tr>
<td>GBM</td>
<td>Glomerular basement membrane</td>
</tr>
<tr>
<td>GFB</td>
<td>Glomerular filtration barrier</td>
</tr>
<tr>
<td>Het</td>
<td>Heterozygous</td>
</tr>
<tr>
<td>HGMD</td>
<td>Human Genome Mutation Database</td>
</tr>
<tr>
<td>HGVS</td>
<td>Human Genome Variation Society</td>
</tr>
<tr>
<td>Homo</td>
<td>Homozygous</td>
</tr>
<tr>
<td>MesPGN</td>
<td>Mesangioproliferative glomerulonephritis</td>
</tr>
<tr>
<td>MGC</td>
<td>Minimal glomerular change</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA (Ribonucleic acid)</td>
</tr>
<tr>
<td>ng</td>
<td>Nanograms</td>
</tr>
<tr>
<td>NPHS2</td>
<td>Nephrosis 2, idiopathic, steroid-resistant (podocin)</td>
</tr>
<tr>
<td>NPSH1</td>
<td>Nephrosis 1, congenital, Finnish type (nephrin)</td>
</tr>
<tr>
<td>NS</td>
<td>Nephrotic syndrome</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PLCE1</td>
<td>Phospholipase C, epsilon 1</td>
</tr>
<tr>
<td>RI</td>
<td>Reliability Index</td>
</tr>
<tr>
<td>SD</td>
<td>Slit diaphragm</td>
</tr>
<tr>
<td>SNPs</td>
<td>Single Nucleotide Polymorphisms</td>
</tr>
<tr>
<td>SRNS</td>
<td>Nephrotic syndrome steroid-resistant</td>
</tr>
<tr>
<td>SSNS</td>
<td>Nephrotic syndrome steroid–sensitive</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris base, acetic acid and EDTA</td>
</tr>
<tr>
<td>T&lt;sub&gt;ann&lt;/sub&gt;</td>
<td>Annealing Temperature</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-EDTA buffer</td>
</tr>
<tr>
<td>TGP</td>
<td>1000 Genomes Project</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>WTI</td>
<td>Wilms tumor 1</td>
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SUMÁRIO

Introdução
A Síndrome Nefrótica é uma doença que leva ao aumento da permeabilidade da barreira de filtração glomerular, sendo uma doença glomerular muito comum na infância. Normalmente caracteriza-se por quatro características clínicas: proteinúria grave, hipoalbuminémia, edema e hiperlipidémia.

A síndrome nefrótica pode ser classificada de acordo com a sua etiologia e com os achados histológicos, aquando da biópsia renal. No que diz respeito à histologia, a grande maioria (> 90%) é idiopática (primária). Uma causa secundária (secundária a doenças sistémicas) acontece muito raramente. Além disso, a síndrome nefrótica também pode ser hereditária.

No que diz respeito à histopatologia, mais de 80% dos doentes apresenta alterações glomerulares mínimas, esclerose segmentar e focal ou esclerose mesangial e difusa. Os casos de proliferação mesangial, caracterizados pela presença de mais de três células mesangiais do lóbulo, encontram-se numa pequena proporção. A maioria dos casos com síndrome nefrótica sensível aos esteroides exibe alterações glomerulares mínimas enquanto os casos com síndrome nefrótica resistente aos esteroides normalmente apresentam esclerose segmentar e focal ou esclerose mesangial e difusa.

A maioria das crianças com síndrome nefrótica é sensível ao tratamento com esteroides, e apresenta um prognóstico favorável a longo prazo. No entanto, aproximadamente 10 a 20% dos casos são resistentes a este tipo de tratamento, apresentando um prognóstico menos favorável e, dos quais, 30 a 40% desenvolvem doença renal crónica.

Na última década, a síndrome nefrótica cortico-resistente em crianças e adultos tem sido associada a defeitos em vários genes. A maioria destes genes codifica proteínas chave na estrutura dos podócitos e do diafragma, participando do seu desenvolvimento e arquitectura estrutural. Mutações nestes genes foram identificadas como estando associadas a dois terços dos doentes com síndrome nefrótica cortico-resistente no primeiro ano de vida. Com o número crescente de genes conhecidos como estando implicados na síndrome nefrótica e a significativa variabilidade fenotípica observada, os testes genéticos são agora uma tarefa mais complexa que precisa ser baseada nas informações clínicas, incluindo o tipo de lesões renais observadas através da histologia.

Assim, actualmente, o diagnóstico genético de rotina em crianças com síndrome nefrótica cortico-resistente não-sindrómica inclui, geralmente, a análise dos genes NPHS1, NPHS2 e
PLCE1. As formas sindrómicas, que são muito menos frequentes, podem ser causadas por mutações no gene WT1. No entanto, mutações neste gene também podem causar síndrome nefrótica cortico-resistente isolada, ou seja, não sindrómica. Como tal, com o objetivo de caracterizar geneticamente um grupo de doentes pediátricos com síndrome nefrótica cortico-resistente, iniciou-se a análise sequencial dos genes NPHS2, NPHS1, WT1 e PLCE1 num conjunto de doentes, seguindo as directrizes internacionais.

**Objetivos**
Num projeto apoiado pela Sociedade Portuguesa de Nefrologia, o nosso principal objetivo residiu na caracterização clínica e genética de um grupo de doentes pediátricos com síndrome nefrótica. Desta forma, propusemo-nos a observar as potenciais associações genótipo-fenótipo de forma a começar a construir um registo clínico e genético para doentes com síndrome nefrótica em Portugal. A caracterização clínica dos doentes foi levada a cabo pelos médicos especialistas do departamento de Nefrologia Pediátrica do Hospital de Santa Maria. As análises genéticas, bem como a interpretação dos resultados, foram realizadas na GenoMed – Instituto de Medicina Molecular – Faculdade de Medicina de Lisboa.

**População e Métodos**
O departamento de Nefrologia Pediátrica do Hospital de Santa Maria procedeu à caracterização clínica dos doentes em estudo. Inicialmente os doentes foram divididos em dois grandes grupos: crianças com esclerose segmentar e focal e crianças com síndrome nefrótica congénita.

Em alguns dos doentes foi realizada a caracterização histológica através de biópsias renais. Esta análise foi realizada anteriormente aos nossos testes genético.

O estudo genético, após extração de ADN a partir de sangue periférico, foi efetuado pela pesquisa sequencial de mutações nos genes NPHS1, NPHS2, WT1 e PLCE1, por PCR e sequenciamento directa.

Foram então analisados dezoito doentes com síndrome nefrótica cortico-resistente, não aparentados, onze com esclerose segmentar e focal e sete com síndrome nefrótica congénita.

Visando a avaliação da patogenicidade das novas mutações encontradas, os resultados obtidos foram analisados através de oito programas diferentes de análise por simulação computacional. Paralelamente analisámos também as mutações previamente descritas na literatura, recorrendo aos programas de análise por simulação computacional, de modo a servir de controlo para a avaliação.
As mutações *missense* foram analisadas por sete diferentes programas de análise por simulação computacional: *SiftBlink, MutPred, SNPeffect, SNPs&Go, HomoloGene, PolyPhen* e *MutationTaster*. As mutações *Frameshift* e de *splicing* foram analisadas por dois programas: *HomoloGene e MutationTaster e Splice Site Prediction e MutationTaster*, respectivamente.

**Resultados e Discussão**
Das dezoito crianças estudadas, encontrámos mutações nos genes *NPHS1, NPHS2* e *WT1* em onze dos casos. Seis dos doentes não apresentaram qualquer mutação nos quatro genes analisados. Uma das crianças revelou uma alteração de significado desconhecido no gene *NPHS2*.

Seguindo as directrizes internacionais, nos sete doentes com síndrome nefrótica congênita, começámos o diagnóstico genético pelo gene *NPHS1*. Deste grupo de doentes, identificámos mutações no gene *NPHS1* em seis dos casos. Nestes seis dos doentes, com síndrome nefrótica congênita, detetáram-se cinco mutações diferentes em homozigotia ou em heterozigotia composta, das quais três são novas mutações. No doente em que não foi identificada qualquer mutação no gene *NPHS1*, continuámos o estudo genético através da análise do gene *NPHS2*, tendo sido identificada uma mutação em homozigotia.

Nos doentes em que o início dos sintomas de síndrome nefrótica aconteceu na infância, a análise genética foi iniciada pelo gene *NPHS2*. Neste grupo de dez doentes, identificámos mutações em três, sendo que duas destas mutações eram novas.

Os restantes sete doentes, que não apresentaram resultado positivo na análise do gene *NPHS2*, foram analisados para o gene *WT1*. Um destes doentes manifestou a presença de uma mutação neste gene.

Um dos casos de síndrome nefrótica cortico-resistente apresentou resultado inconclusivo uma vez que possui uma variante, em heterozigotia, de significado controverso no gene *NPHS2*. Nenhum dos doentes analisados apresentou mutações no gene *PLCE1*.

**Conclusão**
O objetivo primordial deste projeto foi analisar doentes com síndrome nefrótica cortico-resistente de modo a identificar novas mutações nos genes analisados, bem como mutações já reportadas anteriormente, e avaliá-las de acordo com a sua patogenicidade utilizando programas de análise por simulação computacional. Isto torna-se essencial numa altura em que surgem novas metodologias na área da genética humana, uma vez que estes novos métodos identificam uma imensa variedade de alterações novas e, normalmente, de
significado desconhecido. Como tal, é importante dispor de ferramentas simples e fiáveis para a avaliação e interpretação destas novas mutações. O nosso trabalho demonstrou que estes programas de análise por simulação computacional são, então, uma ferramenta que pode ser utilizada no trabalho de rotina. Além disto, o nosso projeto demonstrou a importância de uma equipa multidisciplinar na avaliação desta e de outras doenças: médicos da especialidade, médico geneticista e o laboratório de diagnóstico genético.

Os resultados obtidos, incluindo as novas mutações identificadas, em conjunto com os dados clínicos fornecem um importante apoio para as associações genótipo-fenótipo.

Em Portugal, este é o primeiro projeto que visa a análise clínica e genética de crianças com síndrome nefrótica. Tencionamos portanto construir bases com informações clínicas e genéticas para a síndrome nefrótica.

Para que se possa praticar uma correta caracterização clínica e genética dos doentes, recorrendo às directrizes internacionais e em conjunto com os nossos colegas do departamento de Nefrologia Pediátrica, pretendemos sugerir orientações que possam facilitar o teste clínico e genético de doentes com síndrome nefrótica em Portugal.

Os nossos resultados realçam ainda a importância do teste genético em doentes pediátricos com síndrome nefrótica, permitindo um diagnóstico específico e influenciando as opções terapêuticas. O diagnóstico genético pode também ajudar famílias com crianças afetadas a perceber a causa e a progressão da doença e a possibilidade de intervir em futuras gravidezes.
**RESUMO**

**Introdução:** A síndrome nefrótica cortico-resistente ocorre em cerca de 20% das crianças com síndrome nefrótica e evolui para doença renal crónica estádio 5 em 30 a 40% dos casos. Mutações em vários genes que codificam proteínas estruturais da barreira de filtração glomerular têm sido associadas à patogénese desta doença. A caracterização genética destes casos é essencial para um diagnóstico exato, um tratamento adequado e para possibilitar o diagnóstico pré-natal de famílias em risco.

**Objetivos:** Este projeto visa a caracterização genética de um grupo de doentes pediátricos com síndrome nefrótica cortico-resistente com o objetivo de identificar mutações já descritas ou novas mutações e fazendo eventuais associações genótipo-fenótipo.

**População e métodos:** Os doentes foram clinicamente avaliados pelo departamento de Nefrologia Pediátrica do Hospital de Santa Maria. Foram então analisados 18 doentes com síndrome nefrótica cortico-resistente, não aparentados, 11 com esclerose segmentar e focal e 7 com síndrome nefrótica congénita. O estudo genético foi feito sequencialmente através da pesquisa de mutações nos genes NPHS1, NPHS2, WT1 e PLCE1 por PCR e sequenciação directa. A patogenicidade das mutações encontradas foi analisada utilizando oito programas diferentes de análise por simulação computacional.

**Resultados:** Foram encontradas mutações em 11 doentes. Em 6 dos doentes foram detectadas mutações do gene NPHS1; em 4 dos casos foram identificadas mutações no gene NPHS2 e num dos casos foi identificada uma mutação no gene WT1. Cinco das mutações encontradas não foram descritas previamente. A maioria dos programas de análise por simulação computacional classificou como patogénicas as novas mutações encontradas.

**Conclusões:** Estes resultados realçam a importância do teste genético em doentes pediátricos com síndrome nefrótica, permitindo um diagnóstico específico e influenciando as opções terapêuticas. As análises por simulação computacional, em relação à patogenicidade das mutações, permitem uma simples e fiável avaliação das novas variantes. Estes resultados podem contribuir para estudos de correlação genótipo-fenótipo.

**Palavras-chave:** Síndrome Nefrótico, cortico-resistente, novas mutações, análise por simulação computacional, associações genótipo-fenótipo.
**ABSTRACT**

**Introduction:** Most children with nephrotic syndrome (NS) are steroid-sensitive, however approximately 20% are steroid-resistant (SRNS) of which 30 to 40% develop end-stage renal disease. Inherited structural defects of the glomerular filtration barrier, caused by mutations in various genes, are responsible of a large proportion of these cases. Thus the genetic characterization is essential for the exact diagnosis, the adequate treatment and to provide prenatal diagnosis to families at risk.

**Objectives:** The aim of this project was to genetically characterize a group of pediatric patients with SRNS in order to identify novel or already described mutations and investigate genotype-phenotype associations.

**Patients and methods:** Patients were clinically characterized at the Department of Pediatric Nephrology at Hospital de Santa Maria. 18 unrelated patients with SRNS were analyzed, 11 with focal segmental sclerosis and 7 with congenital nephrotic syndrome. Genetic study was carried out stepwise in the *NPHS1, NPHS2, WT1* and *PLCE1* genes by PCR and direct sequencing. Mutations were analyzed for pathogenicity using eight different *in silico* programs.

**Results:** Were detected mutations in 11 cases. Of these, 6 cases showed mutations in *NPHS1* gene, 4 cases with mutations in *NPHS2* gene and 1 case carried a mutation in *WT1*. No patients showed mutations in the *PLCE1* gene. Five of the mutations are novel: 3 in *NPHS1* and 2 in *NPHS2*. Novel mutations were predicted as pathogenic with most *in silico* programs.

**Conclusions:** Our results emphasize the importance of genetic testing in pediatric patients with NS, enabling a specific diagnosis and influencing therapeutic options. The *in silico* programs allowed a simple and reliable evaluation of the novel variants. Thus they turned out to be useful tools in the daily diagnostics. Our results of 18 patients can contribute to studies of genotype-phenotype correlation in NS.

**Keywords:** Nephrotic Syndrome, steroid-resistant, *in silico* tests, novel mutations, genotype-phenotype associations.
**INTRODUCTION**

**What is Nephrotic Syndrome?**
Nephrotic syndrome (NS) is a clinical state caused by various renal diseases that increase the permeability across the glomerular filtration barrier. It is classically characterized by four clinical features: severe proteinuria, hypoalbuminemia (serum albumin <2.5g/dL), hyperlipidemia (serum cholesterol >200 mg/dL) and edema and is one of the most common glomerular diseases in childhood.\[1-12\]

Most children with NS are steroid–sensitive (SSNS) with a favourable long-term prognosis. Approximately 10 to 20% are NS are steroid–resistant (SRNS) with a worse prognosis and 30 to 40% of them develop end-stage renal disease (ESRD) after a 10 years follow-up.\[1, 5-7, 9, 10, 12-19\]

**Pathophysiology**
Ultrafiltration of blood during formation of the primary urine in the glomeruli (Figure 1) is one of the crucial functions of the human kidney.\[10, 20, 21\]

Urinary loss of macromolecules, such as albumin, reflect a dysfunction of the highly permselective glomerular filtration barrier (GFB). The GFB (Figure 1) consists of three interacting layers: 1) the glomerular fenestrated endothelium, 2) the glomerular basement membrane (GBM) and 3) the podocytes, with their interdigitated foot processes that are interconnected by the slit diaphragm (SD), a multiprotein structural and signalling complex, which plays the critical role for maintaining the barrier function of glomerular capillary wall.\[2, 3, 5, 6, 8, 10-12, 20-26\]

In fact, the majority of glomerular diseases is characterized by alterations in the molecular composition of the SD and a reorganization of foot process structure with fusion and effacement.\[22, 26\] Podocytes provide structural support to the glomerular capillaries and synthesize the proteins of the SD and many extracellular matrix components of the GBM.\[2, 3, 25, 27\]

The profound morphologic changes occurring during NS may be reversible in cases without a primary podocyte defect.\[22, 25, 28, 29\] Podocyte injury leads to effacement, flattening and retraction of the podocyte foot processes, which is the major structural correlate of nephrotic proteinuria.\[3, 10, 21, 22\] This change in podocyte shape leads to proteinuria and requires rearrangement of the actin cytoskeleton, a process that is typically reversible with glucocorticoid therapy in minimal glomerular change (MGC) but irreversible and progressive in focal and segmental glomerulosclerosis (FSGS).\[3, 22\]
NS can be classified according to aetiology and also according to the histological finding. According to aetiology, the large majority (>90%) of NS is idiopathic (primary); a secondary cause (secondary to systemic diseases) is seen rarely. \cite{1, 6, 19} Besides, NS can be hereditary. \cite{1, 6, 13, 14, 30} According to the histological finding, in over 80% of the patients, the histology shows insignificant glomerular abnormalities on light microscopy, termed MGC. Electron microscopy reveals effacement of podocytes foot processes with disruption and disorganization of actin filaments. Mesangial proliferation, characterized by the presence of >3 cells per mesangial lobule, is found in a small proportion. \cite{1, 6} Whereas most of the cases with SSNS exhibit MGC distinguished by normal glomeruli at light microscopy and diffuse podocyte foot-process effacement on electron microscopy, the cases with SRNS typically show either FSGS or diffuse mesangial sclerosis (DMS). \cite{2, 9, 10, 15, 16, 19, 25, 31} The underlying pathology in patients with SRNS, and in 5-10% cases with SSNS, is FSGS. Based on location of sclerosis, various subtypes are: (i) tip lesions, (ii) cellular variant, (iii) perihilar lesions, and (iv) FSGS not otherwise specified alterations. \cite{1, 14} Besides this, the histology in patients with SRNS shows MGC (30-40% of the cases), and mesangiproliferative glomerulonephritis (MesPGN) membranous nephropathy, IgA nephropathy or amyloidosis in about 15%. \cite{1, 3, 15, 16} Several syndromic forms of nephrotic syndrome are associated with DMS. \cite{1}
Figure 1: Normal Glomerulus and Glomerular Filtration Barrier. Panel A: Schematic representation of one normal glomerulus with all of its components. Panel B: The glomerular capillary wall and selected components of the filtration barrier. On the urinary side, the interdigitating podocyte foot processes are aligned in regular arrays separated by filtration slit diaphragms located above the glomerular basement membrane. The fenestrated glomerular endothelium is present at the blood interface. The inset diagrams show some of the molecules that make up the slit diaphragm (above) and the basal surface of the podocyte (below). Adapted from “Focal Segmental Glomerulosclerosis” by V. D. D’Agati, F. J. Kaskel, R. J. Falk; 2011; The New England Journal of Medicine; 365:2398-411.

**Genetics**

During the past decade, defects in various genes have been associated with the development of SRNS in children and adults.¹,⁶ Most of these genes, encoding key podocyte proteins that constitute the SD or the podocyte cytoskeleton, participate in the development and structural architecture of these glomerular visceral epithelial cells.¹, 2, 3, 6, 13, 17, 22, 32 Mutations in these genes have been found in two third of the patients that develop SRNS in the first year of life.¹, 13, 21, 28

With the rapidly increasing number of genes known to be implicated in NS and the significant phenotypic variability observed, genetic testing is now a more complex task which needs to be based on different clinical information, including – when available – the type of renal histological lesions.¹³ Genetic mutations are present in 10–20 % patients with sporadic SRNS and in a higher proportion of patients with familial nephrotic syndrome.¹

To date, mutations in several genes have been implicated in different forms of nonsyndromic SRNS. The most commonly affected genes are *NPHS1*, *NPHS2* and *PLCE1* (Table 1). Syndromic forms of SRNS, which are far less frequent, but – in rare cases – also isolated...
SRNS in infants and children, may be due to mutations in e.g. \textit{WT1} that is coding a transcriptional factor or in other GBM components. \cite{1, 2, 6, 9, 12, 13, 22, 32, 33}

The main structural elements of the kidney barrier SD are proteins, encoded by two of these genes, namely \textit{NPHS1}, encoding nephrin and \textit{NPSH2}, encoding podocin. \cite{2, 11, 13, 22, 29, 34, 35} A complex between nephrin and podocin seems to be indispensable to maintain podocyte function, namely the structural integrity of the SD. These genes play a critical role in regulating glomerular protein filtration and this way in the pathogenesis of proteinuria. \cite{2, 13, 22, 23, 25, 26, 28, 31}

\textbf{Table 1:} Genes involved in hereditary forms of Nephrotic Syndrome \cite{1, 2, 14, 22}

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Inheritance</th>
<th>Protein</th>
<th>Function</th>
<th>Phenotype or Syndrome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Slit-Diaphragm protein complex</td>
<td>Main component of the SD. Anchors the SD to the actin cytoskeleton. Modulate signalling events related with actin cytoskeleton dynamics, cell polarity and survival.</td>
<td>CNS (Finnish type). Early-onset SRNS in cases carrying at least one mild mutation.</td>
</tr>
<tr>
<td>NPHS1</td>
<td>19q13.1</td>
<td>AR</td>
<td>Nephrin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NPSH2</td>
<td>1q25-31</td>
<td>AR</td>
<td>Podocin</td>
<td>Scaffold protein linking plasma membrane to the actin cytoskeleton. Modulates mechanosensation.</td>
<td>CNS. Early and late-onset AR SRNS. Juvenile and adult SRNS in cases bearing the R229Q variant in compound heterozygous state with a pathogenic mutation. FSGS.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Phospholipase Cε</td>
<td>Involved in cell junction signalling and glomerular development.</td>
<td>CNS. Early-onset SRNS with DMS or FSGS.</td>
</tr>
<tr>
<td>PLCE1</td>
<td>10q23</td>
<td>AR</td>
<td>Phospholipase Cε</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wilms’ tumor 1</td>
<td>Zinc finger transcription factor that functions both as a tumor suppressor and as a critical regulator of kidney and gonadal development.</td>
<td>Early-onset SRNS. Denys–Drash or Frasier syndrome. WAGR syndrome. Isolated FSGS and DMS.</td>
</tr>
</tbody>
</table>

\textbf{Nephrin}

Nephrin (Figure 2), that contains 29 exons, is a transmembrane protein of the immunoglobulin superfamily with eight extracellular Ig-like motifs. Each immunoglobulin motif is encoded by two exons, except motif Ig2, which is encoded by three exons. Exons 22 and 23 code for a fibronectin type III–like domain, and exon 24 codes for the transmembrane domain. Exons 25-29 encode the putative cytosolic domain and the 3’UTR. Nephrin is predominantly expressed in the podocytes. \cite{1–3, 6, 8, 10, 11, 13, 18, 20, 22-26, 28, 30, 34, 36-38, 41}
Nephrin has structural as well as signalling functions and that signal can be augmented by the complex formed between nephrin and podocin. [6, 11, 18, 20, 22-25, 27, 28, 38, 39] The specific location of nephrin in the SD suggests that it is the principle component of the SD structure. [8, 20, 36] Therefore, mutations in this gene might result in a disruption of the GFB and in consecutive massive protein loss. Defects in this gene are the main cause for the congenital NS (CNS). [5, 9, 10, 19, 20, 23, 25, 26, 28, 34-38]

**Podocin**

Another essential protein of the SD complex is podocin (Figure 3), an integral protein, homologous to the band-7 stomatin family. [6, 19, 21, 22, 23, 25, 29, 31, 41, 42] Podocin has a predicted hairpin-like structure, is expressed at the SD part of the podocytes, with both protein termini (N- and C-) on the cytosolic side. It has been shown to be highly expressed in fetal glomeruli. [6, 10, 11, 19, 21, 22, 23, 25, 28, 31, 40, 41, 42] Podocin localizes to the podocyte foot process membrane, in the place of anchorage of the slit diaphragm. [6, 22, 25] Podocin is normally targeted to the plasma membrane via the classical endoplasmic reticulum (ER) pathway. [21, 25] Podocin may probably have functions as a stabilizing structure with signalling potential. [22, 29, 42] Stabilization of the SD structural assembly and functional integrity is essential for normal glomerular permselectivity. [10, 25, 29, 31] Mutations of podocin result in a dysfunction of the GFB and are responsible for the recessive form of SRNS. [5, 6, 9-12, 18, 19, 22, 25-27, 31, 34, 38, 40]
Phospholipase Cε1

The PLCE1 gene (Figure 4) encodes PLCε1, which is a phospholipase protein that catalyses hydrolysis of membrane phospholipids to generate the second messenger molecules, thus initiating intracellular pathways of cell growth and differentiation. [2, 12, 13, 22, 33, 26] PLCε1 has a widespread distribution, but within the kidney the PLCε1 protein is enriched in glomeruli and localizes in the cytoplasm of the podocyte cell body and both major and intermediate processes. [12, 26] How a PLCE1 gene defect results in changes in the glomerular nephrotic syndrome is still unknown. One possible explanation is that PLCε1 interacts with GTPase-activating protein, which is known to interact with nephrin. Perturbations of this normal interaction would have a downstream effect including the subsequent interaction of GTPase-activating protein with nephrin. [22, 33] It has also been suggested that the absence of PLCε1 may halt kidney development at the capillary loop stage leading to the morphological phenotype of DMS. [12, 26] Due to its functional connection, mutations in PLCE1 are associated with a reduction in the expression of nephrin and podocin. [12, 26]

Mutations in this gene lead to proteinuria in the early childhood and the histological findings depend on the type of the mutations found: DMS (truncating mutations) or FSGS (missense mutations). The majority of children with mutations in this gene have a poor prognosis. [12, 26]

Wilms’ tumor 1

The WT1 gene (Figure 5) includes 10 exons and encodes a protein that will originate four zinc finger motifs in the carboxyl-terminal portion form the DNA binding domain. [35, 43] This gene
has one alternative translational start site (CTG) that is located 204 bp upstream of the major ATG site, creating an isoform with 68 additional amino acids (AA). It also has two alternatively spliced exons, one inserting or excluding exon 5, which encodes 17AA and the other affecting exon 9 via insertion or exclusion of three AA [lysine-threonine-serine (KTS)] between the third and forth zinc finger. The addition of a 68AA from an alternative translational start site has been demonstrated to have little effect on the transcriptional activity of the protein. The alternatively spliced exon 5 has been suggested to increase the repressing effect of WT1 on some promoters. This alternative exon 5 has been found only in mammalian species, indicating that it has been adopted by WT1 at a later stage in evolution.

Mutations in WT1 gene are the cause of Wilms’ tumor and syndromic or isolated SRNS in infants and children. In the fetal kidney, WT1 is expressed in the metanephric mesenchyme, renal vesicles, and is normally also expressed in podocytes from early steps of nephrogenesis. In adult life, the WT1 expression is restricted to the podocytes. Transcriptional activation of NPHS1 and upregulation of NPHS1 mRNA by WT1 have been shown and explained how mutations in WT1 can result in NS. Dominant mutations in WT1 gene can lead to Denys–Drash or Fraiser syndromes, with the main clinical characteristics of male pseudohermaphroditism, progressive glomerulopathy, mesangial sclerosis, nephroblastoma and Wilms tumor, but they can also cause isolated SRNS. In Fraiser syndrome, gonadal development in 46,XX female patients is normal but a complete male to female gender reversal occurs in 46,XY patients. WT1 mutations associated with NS are restricted to exons 8 and 9, which represent a sort of hot-spot that may be easily investigated.

Figure 5: Organization of the WT1 gene and basic structure of the proteins. The WT1 gene consists of 10 exons. Adapted from “A Role for the Wilms’ Tumor Protein WT1 in Organ Development” by Scholz H., Kirschner K. M.; Physiology, 2005; 20:54-59.
Nonsyndromic steroid-resistant nephrotic syndrome

Congenital nephrotic syndrome
The most common type of CNS is congenital nephrotic syndrome of the Finnish type (CNF), a recessively inherited disorder characterized by massive proteinuria detectable at birth, but frequently starting in utero, a large placenta, marked edema, within the first 3 months of life. Characteristic radial dilatations of the proximal tubules are detected more frequently after 3 months of age, but, in some cases they have also been identified already in utero. \[^{5, 6, 8, 9, 13, 20, 22, 23, 24, 28, 30, 37-39, 41}\] NPHS1 has been identified as the major gene involved in CNF. Although NPHS1 is the main gene that has been identified in patients presenting NS in the first 3 months of life, it has also been shown that CNS may be caused by mutations in other genes, including NPHS2. \[^{2, 5, 6, 8, 9, 13, 18, 23, 30, 37-39, 41}\]

Mutations in the PLCE1 and WT1 genes have also been detected in patients presenting isolated CNS with DMS on renal histology. \[^{13, 26, 27, 38, 39}\] These observations suggested that, for patients presenting non-syndromic CNS, the NPHS1 gene should be tested first. \[^{13}\] Molecular analysis of NPHS2 should be the next step whenever mutations in NPHS1 are not detected. \[^{13}\] Patients presenting later in the congenital period (particularly if renal biopsy shows FSGS or MGC) should probably be initially screened for NPHS2 mutations, followed by NPHS1. \[^{13}\] In cases for which renal histological findings are available and DMS is determined, genetic testing of the WT1 and PLCE1 genes should initially be performed. \[^{13, 22, 26, 33}\]

Infantile and childhood nephrotic syndrome
The term infantile NS has been proposed for patients that develop NS between the ages of 4 and 12 months. \[^{13}\] The term childhood NS can be divided in early or late childhood onset and refers the children that develop NS between the ages of 13 months and 5 years or between 6 to 12 years, respectively. \[^{9}\] NPHS2 mutations are responsible for most of these cases. Mutations in this gene occur in about 30-46% of familial and 11–19% of sporadic SRNS cases; patients with mutations in NPHS2 typically present NS from birth to 6 years of age and reach ESRD before the end of their first decade of life. \[^{12, 13}\] The screening for NPHS2 mutations in patients presenting MGC, FSGS or MesPGN on renal biopsies should be performed prior to the initiation of additional – potentially deleterious – therapy. \[^{2, 5, 13, 18}\]
It has recently been shown that mutations in *NPHS1* also account for a non negligible proportion of infantile and childhood-onset SRNS cases with MGC, FSGS or MesPGN on renal biopsies.\(^2,13,18\)

In addition to *NPHS2* and *NPHS1*, *PLCE1* is also involved in some infantile and childhood-onset SRNS cases and is the main gene causing DMS (10-50% of the cases). *PLCE1* mutations have been detected in 28.6% of families with isolated DMS, with the clinical onset of reported cases of DMS varying from few days of life to 4 years of age and all patients having truncating mutations. Nevertheless, *PLCE1* mutations remain an infrequent cause of FSGS (idiopathic or hereditary) – 12% of the cases.\(^2,9,13\)

*WT1* mutations account for about 9% of patients with non familial isolated SRNS, and they have been identified in patients with isolated DMS, with a clinical onset varying from a few days of life up to 2 years of age, as well as in isolated FSGS (1–14 years of age).\(^13,45\)

Based on these observations, *NPHS2* followed by *NPHS1* remain the first genes to be tested in nonsyndromic patients presenting SRNS associated with minimal glomerular changes/FSGS in the infantile or childhood period. In the remaining patients with the same histological lesions, genetic testing for *WT1* mutations (exons 8 and 9 in phenotypically female patients) should be performed, while screening for *PLCE1* mutations may be considered in some – mainly familial – cases.\(^13\)
OBJECTIVES

In a project supported by the Portuguese Society of Nephrology, we aimed to clinically and genetically characterize a group of pediatric patients with NS. This way we proposed to describe novel genetic variants associated to NS, observe potential genotype-phenotype associations and to start to build up a nation-wide clinical-genetic registry for NS patients in Portugal. This is the first initiative, as such, in Portugal, until now.

To fulfil our aims, we created a study group that includes a) medical specialists from the Department of Pediatric Nephrology at the University Hospital Santa Maria, b) a medical geneticist and c) a genetic diagnostic lab – GenoMed – at Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa.

We sequentially screened for mutations following international guidelines (Figure 6) in the NPHS1, NPHS2, WT1 and PLCE1 genes in order to search for already described and for not yet reported mutations.

As part of my Master thesis, I was carrying out the DNA extraction and the mutation screening of the patients and the evaluation of pathogenicity with the in silico programs. Besides, I was discussing the clinical and genetic findings with the clinicians to compare genotypes with the phenotypes of the patients.

**Figure 6:** Genetic approach in children with isolated steroid-resistant nephrotic syndrome. Reprinted from “Hereditary nephrotic syndrome: a systematic approach for genetic testing and a review of associated podocyte gene mutations” by Benoit G, Machuca E, Antignac C.; Pediatric Nephrology, 2010; 25:1621-1632.
**PATIENTS, MATERIAL AND METHODS**

**Patients**
The clinicians of our research team characterized 18 unrelated children with NS (Table 2). From those, twelve were Portuguese and the other six where from Cape Verde, Mozambique or São Tomé and Príncipe. The project was approved by the local Ethical Committee.

From the 18 children who underwent clinical evaluation, seven patients were diagnosed with NS in the first 3 months of life (CNS), one with SRNS and mesangial proliferation with IgM deposits and the other ten cases had SRNS with FSGS.

All patients underwent detailed clinical studies following a standard protocol and a pedigree of each family was drawn. After obtaining signed informed consents from the parents, 5-8 ml peripheral blood sample was collected from the patients, into an EDTA tubes.

**Table 2: Patients who underwent clinical evaluation**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Year of birth</th>
<th>Clinical diagnosis</th>
<th>Age of onset</th>
<th>Country</th>
<th>Genes tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>15318-MS</td>
<td>2008</td>
<td>CNS</td>
<td>1st day (Congenital)</td>
<td>Portugal</td>
<td>1) NPSH1</td>
</tr>
<tr>
<td>15357-MA</td>
<td>2006</td>
<td>CNS</td>
<td>2 months (Congenital)</td>
<td>Cape Verde</td>
<td>1) NPSH1</td>
</tr>
<tr>
<td>15403-LB</td>
<td>2007</td>
<td>CNS</td>
<td>&lt; 1 month (Congenital)</td>
<td>Cape Verde</td>
<td>1) NPSH1</td>
</tr>
<tr>
<td>15739-DC</td>
<td>2004</td>
<td>CNS</td>
<td>1½ months (Congenital)</td>
<td>Portugal</td>
<td>1) NPSH1</td>
</tr>
<tr>
<td>15970-LL</td>
<td>2006</td>
<td>CNS</td>
<td>&lt; 1 month (Congenital)</td>
<td>Portugal</td>
<td>1) NPSH1</td>
</tr>
<tr>
<td>19104-KT</td>
<td>2000</td>
<td>CNS</td>
<td>3 days (Congenital)</td>
<td>Mozambique</td>
<td>1) NPHS1 2) NPSH2</td>
</tr>
<tr>
<td>21080-CN</td>
<td>2000</td>
<td>CNS</td>
<td>&lt; 1 month (Congenital)</td>
<td>Portugal</td>
<td>1) NPHS1</td>
</tr>
<tr>
<td>13338-BM</td>
<td>2004</td>
<td>SRNS + FSGS</td>
<td>4 years (Childhood)</td>
<td>Portugal</td>
<td>1) NPHS2 2) NPSH1 3) WT1 4) PLCE1</td>
</tr>
<tr>
<td>16808-MO</td>
<td>2004</td>
<td>SRNS + FSGS</td>
<td>6 months (Infantile)</td>
<td>Portugal</td>
<td>1) NPHS2 2) NPSH1 3) WT1</td>
</tr>
<tr>
<td>20363-AP</td>
<td>1997</td>
<td>SRNS + FSGS</td>
<td>12 years (Childhood)</td>
<td>Cape Verde</td>
<td>1) NPHS2 2) NPSH1 3) WT1 4) PLCE1</td>
</tr>
<tr>
<td>20949-FR</td>
<td>1992</td>
<td>SRNS + FSGS</td>
<td>6 years (Childhood)</td>
<td>Portugal</td>
<td>1) NPHS2 2) NPSH1 3) WT1 4) PLCE1</td>
</tr>
</tbody>
</table>
DNA Extraction

Genomic DNA was extracted from peripheral blood with an in house method in our laboratory. After the lysis of the blood cells the DNA was ethanol precipitated. This procedure was performed in a Telstar biological safety cabinet class II-A.

The red cell lysis was performed through the addition of the appropriate buffer (Red Cell Lysing Buffer) to the whole blood sample and 20 minutes incubation on ice. The white blood cells were isolated after centrifugation (8 minutes, 2000g) and lysed (white cell lysis buffer). The proteins were precipitated (protein precipitation buffer) and separated from the solution after centrifugation (20 minutes, 2500g). The solubilized DNA was then precipitated with 2-propanol and washed with ethanol (70% v/v). The DNA was resuspended (TE buffer) in a variable volume of buffer, according to the amount of DNA obtained.

DNA Quantification

The DNA solution was quantified using a full spectrum (220-750nm) spectrophotometer, NanoDrop® ND-1000 (NanoDrop® Technologies).

DNA absorbs radiation between 220 and 320nm, presenting maximum absorbance (Abs) at 260nm. Simultaneous measurements at 230 and 280nm were performed, in order to obtain the following ratios:

\[ R_1 = \frac{Abs_{260\text{nm}}}{Abs_{280\text{nm}}} \quad \text{and} \quad R_2 = \frac{Abs_{260\text{nm}}}{Abs_{230\text{nm}}} \]
which relate to DNA solution purity. From the spectrophotometric analysis, values of $R_1 \approx 1.8$ and $1.8 < R_2 < 2.2$ should be observed in order to be considered a high purity DNA solution.\footnote{46}

A low $R_1$ or $R_2$ value could be caused by the presence of proteins, phenol or other reagent associated with the extraction protocol. High $R_1$ value is not indicative of an issue. However, a high $R_2$ value may be the result of making a blank measurement on a dirty pedestal or using an inappropriate solution for the blank measurement.\footnote{46}

After the quantification, the working solutions were diluted from stock (to 100-150ng/µl) and stored at 4°C, while the stock solutions were stored at -20°C.

**Mutation screening**

We screened stepwise for mutations in the *NPHS1* (19q13.1, MIM 602716, NM_004646.3), *NPHS2* (1q25-q31, OMIM 604766, NM_014625.2), *WT1* (11q13, OMIM 607102, NM_024426.4) and *PLCE1* (10q23, OMIM, NM_016341.3) genes. Analyses were carried out with polymerase-chain-reaction (PCR) using flanking intronic primers (Annexes 1 to 4), followed by direct sequencing of the PCR products (Sanger Sequencing, ABI Prism 3100-Avant Capillary Array, 36cm, Applied Biosystems) using a BigDye v3.1 sequence kit (Applied Biosystems). The nomenclature used to describe the genetic variants found in these patients follows the HGVS guidelines.\footnote{47} All coding parts (exons and intron-exon boundaries) of the four genes were analyzed.

**PCR conditions**

Standard PCR protocol was extensively used, with due adjustments in the annealing temperature. We mostly used the PCR based protocols using BioTaq Polymerase as well as respective buffers and magnesium solutions. Briefly, the PCR protocol consists in an initial denaturation step at 96°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, annealing at specific temperature for 30 seconds (Annexes 1 to 4), extension at 72° for 45 seconds, and a final extension step at 72° for 10 minutes.

The dNTPs for the synthesis were provided by *dNTP Mix* (Bio-39028, Bioline) containing nucleotides at a concentration of 25mM each.

The PCR reactions were performed using 100-150ng of template DNA and a non-template control was used. This procedure assures that only the sample DNA is amplified and excludes possible contaminations.

The polymerase used for the vast majority of PCR amplifications was *Biotag™ DNA Polymerase*, according to its efficiency and cost. However, when unspecific PCR products
were observed after the use of standard protocols and its variations (i.e. DMSO, formamide or glycerol addition, magnesium concentration gradient), *Amplitaq Gold*® (4311818, *Applied Biosystems*) was used (Annexes 1 to 4). This enzyme is inactive at low temperatures and is activated after the initial denaturation step, preventing unspecific annealing of primers and consequent unspecific fragment amplification. Magnesium concentration can be decreased when unspecific products are observed, or increased when a low yield PCR product is obtained. These small changes may be made in order to improve the specificity of the PCR in some samples and/or increase the amount of product obtained to facilitate sequencing.

**Gel electrophoresis**

After PCR reaction, all samples and non-template controls were analyzed on agarose gel to check for specificity and contamination. Agarose gels concentrations of 1.5 to 3% can separate fragments of 100 to 1000 bases in size and, according to the size of the fragments being amplified, 2% gels were used (TAE buffer). The size of the fragments was assessed by a molecular size ladder included, GeneRuler™ 100bp DNA Ladder (*Fermentas*). In order to visualize the DNA, the gel was stained with ethidium bromide. This is a DNA intercalator, inserting itself into the spaces between the base pairs of the double helix. In order to see these complexes, we set them under UV light.

**PCR purification**

The PCR products had to be purified as several PCR reaction components, such as excess dNTPs or primers, interfere with the subsequent sequencing reaction.

The purification was performed using a vacuum purification system, *Montage™ MultiScreen™ PCR₉₆ Cleanup Kit* (LSKM PCR50, *Millipore*). The PCR products were transferred to 96 well plates with filter tips and vacuum was used to separate the amplified fragments from the solution. The PCR product was resuspended in Milli-Q water and stored at 4ºC.

**Sanger Sequencing**

The mutations screening was done through the sequencing reactions that were made using the “Big Dye v3.1 Cycle Sequencing kit”. The analyses of the final products, after purification, were made in an automatic sequencer (*ABI Prism 3100-Avant Capillary Array, 36cm, Applied Biosystems*).
The efficiency of the analysis with the sequencer machine depends on a previous purification of the sequencing reaction (Montage™ SEQ96 Sequencing Reaction Cleanup Kit, Millipore) that will exclude the other components, such as salts, primers that were not incorporated during the reaction, or like dNTP’s and ddNTP’s, that could interfere with the fluorescence emitted by the sequence that we want to analyze. The purification was done adding 50µL of Montage™ Sequencing Wash Solution to each sequencing reaction, the solution was transferred to an appropriate purification plate and, after submitting it to vacuum, this procedure was repeated. After the washing step, the Montage™ Injection Solution was added to each well of the purification plate, and the sequencing products were resuspended. The samples were transferred to a 96 well plate and the sample preparation for sequencer analysis was performed by the addition of Hi-Di™ formamide to each sample. The sequencer data was analyzed using the DNA Sequencing Analysis Software™ Version 5.1 (Applied Biosystems) and the final electropherograms were printed and stored. The identification of sequence variants, pathogenic or polymorphic, in the DNA was performed comparing the sequences obtained for each patient to a reference sequence, downloadable through Ensembl website. The software Sequencher™ was used to perform the alignment and comparison of the different sequences, and also notes any discrepancy between the reference sequence and the patient sequence. A molecular report was issued for each patient and was addressed to the Department of Pediatric Nephrology at Hospital de Santa Maria.

**In silico Analysis Programs**

Pathogenicity of the novel missense mutations was analyzed with the following in silico programs: Sift Blink, MutPred Server, SNPeff 4.0, SNPs&GO, PolyPhen-2 and MutationTaster. Novel splice site mutations were evaluated with the Splice Site Prediction program and the MutationTaster software. Novel frameshift mutations were also tested with the MutationTaster software. We carried out multiple sequence alignment of nephrin and podocin in various species with the HomoloGene program.

**HomoloGene**

Genes identified as putative homologues of one another during the construction of HomoloGene. 

The input for HomoloGene processing consists of the proteins from the input organisms. These sequences are compared to one another and then are matched up and put into groups.
using a tree built from sequence similarity to guide the process, where closer related organisms are matched up first, and then further organisms are added as the tree is traversed toward the root. The protein alignments are mapped back to their corresponding DNA sequences. \[48\]

Remaining sequences are matched up by using an algorithm for maximizing the score globally, rather than locally, in a bipartite matching. Cutoffs on bits per position and Ks values are set to prevent unlikely "orthologs" from being grouped together. These cutoffs are calculated based on the respective score distribution for the given groups of organisms. Paralogs are identified by finding sequences that are closer within species than other species. \[48\]

**Sift Blink**

SIFT predicts whether an amino acid substitution will affect protein function on the basis of the degree to which the amino acid residue is conserved during evolution, thereafter designating changes as either tolerated or deleterious. \[49\]

This program is a sequence homology-based tool that sorts intolerant from tolerant amino acid substitutions and predicts whether an amino acid substitution in a protein will have a phenotypic effect. SIFT is based on the premise that protein evolution is correlated with protein function. Positions important for function should be conserved in an alignment of the protein family, whereas unimportant positions should appear diverse in an alignment. \[49\]

SIFT takes a query sequence and uses multiple alignment information to predict tolerated and deleterious substitutions for every position of the query sequence. SIFT is a multistep procedure that (1) searches for similar sequences, (2) chooses closely related sequences that may share similar function to the query sequence, (3) obtains the alignment of these chosen sequences, and (4) calculates normalized probabilities for all possible substitutions from the alignment. Positions with normalized probabilities < 0,05 are predicted to be deleterious and ≥ 0,05 are predicted to be tolerated. \[49\]

**MutPred Server**

This program classifies an AA substitution as disease-associated or neutral in human. MutPred is based on SIFT and was trained using neutral Single Nucleotide Polymorphisms (SNPs) from Swiss-Prot together with deleterious mutations from the Human Genome Mutation Database (HGMD), thereby greatly increasing its reliability. It gives each variant a general score of the probability that the amino acid substitution is disease-
associated/deleterious (0-1, where 0.5-0.75 is considered mildly deleterious and >0.75 is considered deleterious). [14, 50]

The output contains a general score (g) i.e., the probability that the amino acid substitution is deleterious/disease-associated, and top 5 property scores (p), where p is the P-value that certain structural and functional properties are impacted. [50]

**SNPeffect 4.0**

SNPeffect primarily focuses on the molecular characterization and annotation of disease and polymorphism variants in the human proteome. This program provide a detailed variant analysis using our tools such as: [51]

→ TANGO: an algorithm that detects aggregation-prone regions in protein sequences by analyzing the hydrophobicity and beta-sheet forming propensity. [51]

→ WALTZ: an algorithm that accurately and specifically predicts amyloid-forming regions in protein sequences. It is thus more specific in terms of aggregate morphology than TANGO. [51]

→ LIMBO: a chaperone binding site predictor for the Hsp70 chaperones, trained from peptide binding data and structural modeling. Accurate prediction of Hsp70 binding sites is an essential prerequisite to understand the precise function of these chaperones and the properties of its substrate proteins. [51]

→ FoldX: if structural information is available, the empirical protein design forcefield FoldX is used to calculate the difference in free energy of the mutation.

SNPeffect also prints out a decision whether the mutation is stabilizing or destabilizing the structure. To obtain structural information, they first check in the UniProt cross-references whether a protein data bank structure is available for the protein and whether the variant position is actually solved in the structure. [51]

Further, SNPeffect holds per-variant annotations on functional sites, structural features and post-translational modification. [51]

**SNPs&GO**

The genetic basis of human variability is mainly due to SNPs. The most investigated SNPs are missense mutations resulting in residue substitutions in the protein. [52]

SNPs&GO was trained on a set of 38460 mutations and tested with a cross-validation procedure. Recently SNPs&GO was also tested by another laboratory and scored among the best predictors available. [52]
The server works with different algorithms as PANTHER, which the predicted probability of deleterious mutation, the frequencies of the wild-type and mutated residue and the number of independent counts or PhD-SNP method, which takes in input the first 45 elements vector encoding for the sequence and profile information and gives the sequence and profile at the mutated position. For a given protein, its sequence profile features are extracted from a BLAST search output. From the output they evaluate the frequency of the wild type and mutated residues. \[^{[52]}\]

**PolyPhen-2**

PolyPhen designates variants as either probably or possibly damaging or benign on the basis of sequence annotation, sequence alignment, and structural parameters. \[^{[18]}\]

PolyPhen-2 is a software tool for predicting damaging effects of missense mutations that uses eight sequence-based and three structure-based predictive features, which were selected automatically by an iterative greedy algorithm. \[^{[53]}\]

The majority of these features involve comparison of a property of the wild-type allele and the corresponding property of the mutant allele. The alignment pipeline selects a set of homologous sequences using a clustering algorithm and then constructs and refines its multiple alignments. The most informative predictive features characterize how likely the two human alleles are to occupy the site given the pattern of amino acid replacements in the multiple-sequence alignment; and how distant the protein harboring the first deviation from the human wild-type allele is from the human protein. The functional importance of an allele replacement is predicted from its individual features by a naive Bayes\(^1\) classifier. This program compiled the first pair, HumDiv, from all 3155 damaging alleles annotated in the UniProt database as causing human Mendelian diseases and affecting protein stability or function, together with 6321 differences between human proteins and their closely related mammalian homologs, assumed to be non-damaging. The second pair, HumVar, consists of all the 13032 human disease-causing mutations from UniProt and 8946 human non-synonymous SNPs without annotated involvement in disease, which we treated as non-damaging. For a false positive rate of 20%, PolyPhen-2 achieved true positive prediction rates of 92% and 73% on HumDiv and HumVar datasets, respectively. \[^{[53]}\]

PolyPhen-2 calculates the naive Bayes posterior probability that a given mutation is damaging and reports estimates of false positive (the chance that the mutation is classified as damaging

\[^1\] Probabilistic classifier based on applying Baye’s Theorem \(P(A|B) = \frac{P(B|A)P(A)}{P(B)}\).
when it is in fact non-damaging) and true positive (the chance that the mutation is classified as damaging when it is indeed damaging) rates. A mutation is also appraised qualitatively, as benign, possibly damaging or probably damaging. [53]

**MutationTaster**

MutationTaster also employs a Bayes classifier to eventually predict the disease potential of an alteration. It calculates probabilities for the alteration to be either a disease mutation or a harmless polymorphism. For this prediction, the frequencies of all single features for known disease mutations/polymorphisms were studied in a large training set composed by more than 390,000 known disease mutations from HGMD Professional and more than 6,800,000 harmless SNPs and Indel polymorphisms from the 1000 Genomes Project (TGP). [54]

MutationTaster provide three different models aimed at different types of alterations: 1) silent alterations (non-synonymous or intronic), 2) alterations leading to the substitution/insertion/deletion of a single amino acid or 3) more complex changes of the amino acid sequence (e.g. mutations introducing a premature stop codon). [54]

The score of the amino acid changes is taken from an amino acid substitution matrix which takes into account the physico-chemical characteristics of amino acids and scores substitutions according to the degree of difference between the original and the new amino acid. Scores may range from 0,0 to 215. Since this matrix does not provide values for an amino acid insertion/deletion, no score is given in such cases. [54]

If a variant is marked as probable-pathogenic or pathogenic in ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/), it is automatically predicted to be disease-causing. [54]

Whenever an HGMD public disease mutation is found at the same position as a variant, this will be written in the summary. [54]

phastCons and phyloP are both methods to determine the grade of conservation of a given nucleotide. [54]

phastCons values vary between 0 and 1 and reflect the probability that each nucleotide belongs to a conserved element, based on the multiple alignment of genome sequences of 46 different species (the closer the value is to 1, the more probable the nucleotide is conserved). By contrast, phyloP (values between -14 and +6) separately measures conservation at individual columns, ignoring the effects of their neighbors. Sites predicted to be conserved are assigned positive scores, while sites predicted to be fast-evolving are assigned negative scores. [54]
MutationTaster determines the position of this splice site change relative to intron/exon borders: if a loss/decrease of a splice site occurs at an intron/exon border or exon/intron border, this will be taken for a "real" splice site change. Loss/decrease of splice sites distant from intron/exon (and reverse) borders will be ignored. A gain of a completely new splice site is displayed, if the confidence score of the newly created splice site is greater than 0.3. An increase in an already existing splice site will be displayed if the change in the confidence score is greater than 10%. [54]

The Kozak consensus sequence (gccRccAUGG; R = purine) starts upstream of the start codon (AUG) and plays a major role in the initiation of translation. The purine (R) at position -3 as well as the G in position +4 are highly conserved. The program checks whether for a given alteration a previously strong consensus sequence has been weakened. [54]

For conservation analysis, amino acid or nucleotide sequence homologues of ten other species (chimp, rhesus macaque, mouse, cat, chicken, claw frog, pufferfish, zebrafish, fruitfly, and worm) are aligned with the corresponding human sequence of the gene in question. The status for local nucleotide sequence alignments is either conserved or not conserved. [54]

A protein feature might get lost if a whole exon is skipped due to splice site changes, or if a protein is shortened because of a premature termination codon - in those cases, protein features are indirectly affected. [54]

**Splice Site Prediction**

Splice sites are the key signal sequences that determine the boundaries of exons. A method for splice site detection should, ideally, be based on a complete understanding of the complex eukaryotic splicing process. This program uses a backpropagation feedforward neural network with one layer of hidden units to recognize 5' and 3' splice sites. They only consider genes that have constraint consensus splice sites, i.e., GT' for the 5' and AG' for the 3' splice site. The output of the network is a score between 0 and 1 for a potential splice site. [55]

Scores ranging from 0 to 1 (where 1 is indicative of the presence of an ideal splice site) were assigned and compared between wild-type and mutant sequences to determine the extent to which the mutation is predicted to alter a splice site. [18, 55]
RESULTS AND DISCUSSION

Clinical and histological results
Clinical characterization was carried out in the Department of Pediatric Nephrology at Hospital de Santa Maria. Patients were initially divided in two main groups: children with FSGS and patients with CNS.
In some of the patients histological characterization of kidney biopsy samples was carried out. These analyses were done prior to our genetic screening. Results were shared to us by the clinicians (Table 2).

Genetic results
From the 18 patients, we detected mutations in the \textit{NPHS1}, \textit{NPHS2} and \textit{WT1} genes in 11 cases (see Table 3). No mutations were detected in \textit{PLCE1}. In 6 patients we did not find any mutation in the four genes analysed. In 1 patient, we found an alteration with a controversial significance in the \textit{NPHS2} gene.

Table 3: Results of the genes tested for each patient.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Genetic results</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>\textit{NPHS1}</td>
</tr>
<tr>
<td></td>
<td>AR</td>
</tr>
<tr>
<td>15318-MS</td>
<td>c.1337T&gt;A Het (p.Ile446Asn)</td>
</tr>
<tr>
<td></td>
<td>Reported + c.3387+1G&gt;A Het</td>
</tr>
<tr>
<td>15357-MA</td>
<td>c.2387delG Homo (p.Gly796Aspfs*51)</td>
</tr>
<tr>
<td></td>
<td>New</td>
</tr>
<tr>
<td>15403-LB</td>
<td>c.2387delG Homo (p.Gly796Aspfs*51)</td>
</tr>
<tr>
<td></td>
<td>New</td>
</tr>
<tr>
<td>15739-DC</td>
<td>c.1337T&gt;A Homo (p.Ile446Asn)</td>
</tr>
<tr>
<td></td>
<td>Reported</td>
</tr>
<tr>
<td>15970-LL</td>
<td>c.2143G&gt;C Homo (p.Gly715Arg)</td>
</tr>
<tr>
<td></td>
<td>Reported</td>
</tr>
<tr>
<td>19104-KT</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>21080-CN</td>
<td>c.1757+1G&gt;T Homo</td>
</tr>
<tr>
<td></td>
<td>New</td>
</tr>
<tr>
<td>13338-BM</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>16808-MO</td>
<td>Negative</td>
</tr>
<tr>
<td>20363-AP</td>
<td>Negative</td>
</tr>
<tr>
<td>20949-FR</td>
<td>Negative</td>
</tr>
<tr>
<td>20980-PF</td>
<td>Negative</td>
</tr>
<tr>
<td>21075-MR</td>
<td>-</td>
</tr>
<tr>
<td>21246-AF</td>
<td>Negative</td>
</tr>
<tr>
<td>23620-BM</td>
<td>Negative</td>
</tr>
<tr>
<td>32193-LP</td>
<td>-</td>
</tr>
</tbody>
</table>

**Findings in NPHS1 gene**

Following the guidelines (Figure 6), in CNS patients we started the genetic screening by NPHS1 gene. 7 patients were analyzed. From those, we found mutations in 6 cases (Table 3). In these 6 cases we found five different mutations in homozygous or compound heterozygous state. Three of these mutations are novel: one deletion causing a frameshift and a premature stop codon and two splice site mutations. The other two were missense mutations that were already reported in other cases.

One of the missense mutation is a T to A substitution at position c.1337, leading to an AA change at codon 446, from an Isoleucine to an Asparagine (p.Ile446Asn). This mutation was identified in two different patients, one time in homozygous state and in the other case in compound heterozygous together with a novel splice site mutation.

The other missense mutation is a G to C substitution in homozygous state at position c. 2143, leading to a Glycine to Arginine AA change at the codon 715 (p.Gly715Arg). This mutation was found in only one patient.

The frameshift mutation was caused by a deletion of a G nucleotide at position c.2387. This deletion leads to a change in the reading frame that starts at codon 796. The mutation causes a
Glycine to Aspartic Acid substitution and a premature STOP codon 51 triplets after the first affected AA (p.Gly796Aspfs*51). This mutation was present in homozygous state in two of the seven cases with CNS.

According to the splice site mutations, one was found in intron 13 in homozygous state in one of the patients. The mutation resulted in a G to T substitution at position c.1757+1. The other splice site mutation, which was found in compound heterozygosity together with the p.Ile446Asn in one patient, was a G to A substitution at position c.3387+1, in intron 26.

According to the patient with CNS, that showed a negative result for NPHS1 gene, we continued the genetic diagnosis with the NPHS2 gene.

**Findings in NPHS2 gene**

In patients with infantile or childhood onset NS, we started the genetic analysis with the NPHS2 gene. From the 10 cases of this group, three patients carried three different mutations in NPHS2. Two of these mutations were novel: one missense and one frameshift. The other one is an already describe variant.

The missense mutation is a G to A substitution at position c.928, leading to an AA change at codon 310, from Glutamic Acid to Lysine (p.Glu310Lys). This mutation was identified in two different patients, in compound heterozygosity, together with an already described missense variant. The latter one is a G to A substitution at position c.686 leading to an Arginine to a Glutamine AA change at codon 229 (p.Arg229Gln). To our knowledge, these two patients were not relatives.

The frameshift mutation was caused by a deletion of two adenines at position c.855_856. This deletion leads to a change in the reading frame that starts at codon 286 where an Arginine is changed to a Threonine and leads to a premature STOP codon 17 triplets after (p.Arg286Thrfs*17). This mutation was present in homozygous state in one patient.

We also analyzed this gene in the patient with CNS but without mutations in NPHS1. This patient had a missense mutation in homozygous state: a nucleotide substitution from T to A at position c.779. This substitution leads to an AA change at codon 260, from a Valine to a Glutamic Acid (p.Val260Glu).

For the other seven patients that showed a negative result for this gene we continued the genetic test by studying the WTI gene.
**Findings in WT1 gene**

From the seven patients that we analyzed, one patient, with SRNS and FSGS that had occurred at 6 months of age (infantile onset), carried a splice site mutation in the WT1 gene. This mutation was a G to A substitution at position c.1432+5 (intron 9).

**Pathogenicity of the novel mutations**

In order to investigate the pathogenicity of the novel mutations that we found in these patients, eight different *in silico* programs were performed. All results of the *in silico* analysis are summarized in Table 4. Parallel to this, as a control, *in silico* analysis were also performed for those already reported variants that we detected in the patients.

Missense mutations were analyzed with seven different programs: SiftBlink, MutPred, SNPefffect, SNPs&Go, HomoloGene, PolyPhen and MutationTaster. Frameshift and splice site mutations were tested with two *in silico* programs: HomoloGene and MutationTaster and Splice Site Prediction and MutationTaster, respectively.

In general, the *in silico* programs turned out to be a reliable tool to assess the pathogenic impact of the mutations that were already reported as pathogenic: e.g. the *NPHS2* missense mutation p.Val260Glu was predicted by SiftBlink as a “not tolerated” alteration, affecting the protein function with a score of 0.00; MutPred predicted a 95.8% probability of pathogenicity; SNPefffect algorithm considered that this mutation decreases the aggregation tendency of the protein; SNPs&Go classified this alteration as disease associated with a probability of 84% and with a high score of Reliability Index (RI 7); PolyPhen predicted a probably damaging alteration with a probability of 0.998 and MutationTaster classified it as a disease causing variant. Besides this, multiple sequence alignment of the *NPHS2* AA sequence in different species was carried out and the affected AA turned out to be a highly conserved one: it was present in all of the nine vertebrates *Homo sapiens*, *Pan troglodytes*, *Macaca mulatta*, *Canis lupus familiaris*, *Bos taurus*, *Mus musculus*, *Rattus norvegicus*, *Gallus gallus*, *Danio rerio*.

Based up on these results, these *in silico* programs regarding to the novel mutations (c.1757+1G>T, c.3387+1G>A, p.Gly796Aspfs*51, p.Glu310Lys and p.Arg286Thrfs*17) gave in general results of strong probability of pathogenicity.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation</th>
<th>State</th>
<th>Mutation reported</th>
<th>Cellular Localization</th>
<th>SiftBlink</th>
<th>MutPred</th>
<th>SNP effect</th>
<th>HomoloGene</th>
<th>PolyPhen</th>
<th>Mutation Taster</th>
<th>Splice Site Prediction</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPHS1</td>
<td>c.1337T&gt;A</td>
<td>Het</td>
<td>Reported</td>
<td>Extracellular Ig-like 5</td>
<td>Predict not tolerated AFFECT PROTEIN FUNCTION (score: 0,00)</td>
<td>95.2%</td>
<td>No Effect</td>
<td>Disease RI 6 (78.1%)</td>
<td>Conserved 10/11</td>
<td>Probably damaging, 1,000</td>
<td>Disease causing</td>
</tr>
<tr>
<td></td>
<td>p.Ile446Asn</td>
<td></td>
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<tr>
<td></td>
<td>c.3387+1G&gt;A</td>
<td>Het</td>
<td>Novel</td>
<td>Cytoplasmic</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Disease causing</td>
</tr>
<tr>
<td>NPHS1</td>
<td>c.2387delG</td>
<td>Homo</td>
<td>Novel</td>
<td>Extracellular Ig-like 7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Disease Causing</td>
</tr>
<tr>
<td></td>
<td>p.Gly715Arg</td>
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<tr>
<td></td>
<td>c.2143G&gt;C</td>
<td>Homo</td>
<td>Reported</td>
<td>Extracellular Ig-like 7</td>
<td>Predict not tolerated AFFECT PROTEIN FUNCTION (score: 0,00)</td>
<td>98,1%</td>
<td>No Effect</td>
<td>Disease RI 7 (77%)</td>
<td>Conserved 11/11</td>
<td>Probably damaging, 1,000</td>
<td>Disease Causing</td>
</tr>
<tr>
<td></td>
<td>p.Gly715Arg</td>
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</tr>
<tr>
<td>NPHS1</td>
<td>c.1757+1G&gt;T</td>
<td>Homo</td>
<td>Novel</td>
<td>Extracellular Ig-like 6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Disease causing</td>
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</tr>
<tr>
<td>NPHS2</td>
<td>c.779T&gt;A</td>
<td>Homo</td>
<td>Reported</td>
<td>Cytoplasmic</td>
<td>Predict not tolerated AFFECT PROTEIN FUNCTION (score: 0,00)</td>
<td>95,8%</td>
<td>Decreases the aggregation tendency of the protein</td>
<td>Disease RI 7 (84%)</td>
<td>Conserved 9/9</td>
<td>Probably damaging, 0.998</td>
<td>Disease Causing</td>
</tr>
<tr>
<td></td>
<td>p.Val260Glu</td>
<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>NPHS2</td>
<td>c.686G&gt;A</td>
<td>Het</td>
<td>Reported</td>
<td>Cytoplasmic</td>
<td>Predict tolerated TOLERATED (score: 0,08)</td>
<td>69,8%</td>
<td>No Effect</td>
<td>Neutral RI 0 (49,5%)</td>
<td>Conserved 8/9</td>
<td>Possibly damaging, 0.903</td>
<td>Disease Causing</td>
</tr>
<tr>
<td></td>
<td>p.Arg229Gln</td>
<td></td>
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</table>

Table 4: *In silico* analyses
<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation Details</th>
<th>Allele</th>
<th>Novel/Reported</th>
<th>Location</th>
<th>Predicted Effect</th>
<th>Neutral RI</th>
<th>Conserved</th>
<th>Probably Damaging</th>
<th>Disease Causing</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NPHS2</strong></td>
<td>c.855_856delAA p.Arg286Thrfs*17</td>
<td>Homo</td>
<td>Novel</td>
<td>Cytoplasmic</td>
<td>43.1% No Effect</td>
<td>Neutral RI 3 (34.6%)</td>
<td>Conserved 9/9</td>
<td>Probably damaging 0.992</td>
<td>Disease Causing</td>
</tr>
<tr>
<td><strong>NPHS2</strong></td>
<td>c.59C&gt;T p.Pro20Leu (rs74315344)</td>
<td>Het</td>
<td>Controversial</td>
<td>Cytoplasmic</td>
<td>72.1% No Effect</td>
<td>Neutral RI 8 (8.4%)</td>
<td>Conserved 5/9</td>
<td>Benign 0.098</td>
<td>Disease Causing</td>
</tr>
<tr>
<td><strong>WT1</strong></td>
<td>c.1432+5G&gt;A</td>
<td>Het</td>
<td>Reported</td>
<td>KTS motif</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Disease Causing Splice Site changes</td>
</tr>
</tbody>
</table>

**Predict not tolerated AFFECT PROTEIN FUNCTION (score: 0.03)**

**Predict not tolerated AFFECT PROTEIN FUNCTION (score: 0.01)**
The missense mutation p.Arg229Gln in *NPHS2* represents one of the most frequently reported non-synonymous *NPHS2* variant. It was reported to cause NS in homozygous state with a late onset of the symptoms. On the other hand it was described to cause early onset NS when appearing together with another pathogenic mutation in compound heterozygoty. \[13, 19, 31, 34\] We found this alteration together with a novel missense mutation in exon 8 of *NPHS2* (p.Glu310Lys) in two patients. No other variants in this gene were found in these two cases. The patients were reported to be unrelated: one is from Portugal and the other is from São Tomé and Príncipe. The latter variant, although some of the *in silico* programs did not classify it as pathogenic, leads to a severe alteration in the structure and polarity of the AA (Annex 5). Taken together the change in structure characteristics, the *in silico* programs that predicted this as a pathogenic variant (SiftBlink, PolyPhen and MutationTaster), and the HomoloGene result showing a highly conserved AA, we predicted the mutation as important in causing the disease.

Additionally, the Department of Pediatric Nephrology sent us a patient for genetic testing for *NPHS2* with a juvenile onset of the disease. This patient revealed the presence of a controversial variant (p.Pro20Leu) in heterozygous state. The pathogenicity of this alteration is not clarified: according to the study of Caridi G. et al., this is a variant of unclear functional significance but is not considered as a mutation.\[17\] The paper of Benoit G. *et al.*, described it as a polymorphism.\[13\] However, McCarthy H. J. reported in 2013 this variant as a potentially pathogenic variant.\[14\] According to our *in silico* predictions this substitution is predicted to affect the protein function (SiftBlink, MutPred and Mutation Taster). However, not many pathogenic mutations were reported at this N-terminal part of the protein. The SiftBlink program reported this alteration to affect function with only a very low confidence. Because of this inconclusive result, we plan to start the genetic testing including other genes as suggested by the guidelines (Figure 6).
CONCLUSION AND FUTURE PROSPECTS

The aim of our project was to analyse a group of pediatric patients with SRNS, which were clinically diagnosed at the Hospital Santa Maria, in order to identify already reported and not yet described mutations. The genetic analysis included the NPHS1, NPHS2, WT1 and PLCE1 genes and the mutations found were evaluated according to their pathogenicity through eight different in silico analysis. This is essential in the era where new methods (e.g. Next Generation Sequencing) of genetic testing appear in the daily diagnostics. These novel diagnostic methods identify a wide variety of novel variants or variants with unknown significance, therefore it is important to have simple and reliable tools for the evaluation and interpretation of these mutations. Novel mutations, particularly missense mutations are normally hard to interpret according to their pathogenicity. Our work showed that the above described in silico programs can be used in a daily routine.

This is the first project in Portugal that analysis clinically and genetically children with SRNS. Further we started to create a registry of SRNS patients in Portugal with clinical and genetic characterization. Therefore, with this project we started to fill this gap in Portugal. The recognition of the genetic origin of the disease and the detection of the mutation is useful also clinically, avoiding (or discontinue) unnecessary and aggressive immunosuppressive therapies for these patients, sparing them from the significant side-effects associated with these drugs, to predict the absence of recurrence after transplantation and to provide prenatal diagnosis to families at risk. Then, to be able to get a good clinical and genetic characterization of the patients, using international guidelines together with the opinion of our colleagues in the Nephrology department, we aim to elaborate suggestions of guidelines that can improve the clinical and genetic testing of NS patients in Portugal.

Our results, together with the clinical data, are adding more information about genotype-phenotype correlations. The phenotypic variation of the disease will be used as the basis to define guidelines for genetic testing. On the other hand, our results point out the importance of multidisciplinary collaboration between medical specialists, medical geneticists, and the genetic diagnostic lab.

Our results emphasize the importance of genetic testing in pediatric patients with NS, enabling a specific diagnosis and influencing therapeutic options.

This project may assure a better understanding of the basic mechanisms of NS and may contribute to a better, earlier and more targeted therapeutic approach and can contribute to
novel drug discovering efforts. Thus we can transpose basic research into clinical application to individual based medical treatment.

Our project makes an important contribution to the establishment of a Portugal-wide reference center for genetic research on NS. No such initiative has been stated until now.


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ANNEXES

Annex 1: Specific intronic primers and PCR conditions of the \textit{NPHS1} gene and thermocycler conditions.

Not available.

Annex 2: Specific intronic primers and PCR conditions of the \textit{NPHS2} gene and thermocycler conditions.

Not available.

Annex 3: Specific intronic primers and PCR conditions of the \textit{WT1} gene and thermocycler conditions.

Not available.

Annex 4: Specific intronic primers and PCR conditions of the \textit{PLCE1} gene and thermocycler conditions.

Not available.
Annex 5: Genetic Code and amino acids properties