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COORDINATORE Prof. Marco Matucci Cerinic

Next-generation sequencing per la diagnosi personalizzata delle nefropatie ereditarie

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Dottorando
Dott. Francesca Becherucci

Tutore
Prof. Paola Romagnani

Coordinatore
Prof. Marco Matucci Cerinic

Dottorato di Ricerca in Scienze Cliniche
Dipartimento di Medicina Sperimentale e Clinica
Il Coordinatore
Prof. Marco Matucci Cerinic

UNIVERSITA' DEGLI STUDI DI FIRENZE
Dottorato di Scienze Biomediche Sperimentali e Cliniche

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To the dreamers

"If we are to make reality endurable, we must all nourish a fantasy or two"

Marcel Proust

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Introduction

Chronic kidney disease in children and adolescents

Chronic kidney disease (CKD) is a major health problem worldwide with increasing incidence and prevalence that is threatening to bring on the onset of a real 'epidemic' (Lysaght MJ, 2012) (United States Renal Data System, 2012) (Schaefer B, 2012) (Schieppati A, 2005) (Brück K, 2015) (Becherucci F, 2016). Independent of the initial cause, CKD is a clinical syndrome characterized by a gradual loss of kidney function over time (group., 2013). Indeed, progression of CKD to end-stage renal disease (ESRD) requires dialysis or kidney transplantation for survival. Data coming from epidemiological studies in adults provide the dramatic evidence that ESRD represents the 'tip of the iceberg' of CKD and suggest that the number patients with earlier stages of the disease are likely to exceed those reaching ESRD by as much as 50 times (Coresh J, 2003). The same consideration could probably be applied to the pediatric population.

The incidence of pediatric CKD rose slowly during the 1980s, then marginally until the first decade of the 21st century. Almost in parallel, the prevalence of the disease has significantly increased since survival and treatment of CKD have markedly improved also in children (Becherucci F, 2016). Epidemiologic data on CKD probably underestimate its real incidence and prevalence since CKD is often clinically asymptomatic, especially in earlier stages, making the epidemiology of CKD very difficult to study. Despite these limitations, the

pediatric incidence of CKD in Europe and other western countries is reported to be around 11–12 *per* million of age-related population (pmarp) for stages 3–5, while the prevalence is ~55–60 pmarp (Becherucci F, 2016). Besides this, no precise data on the incidence and prevalence of pre-terminal CKD are available for the majority of countries (Warady BA, 2007).

The Kidney Disease: Improving Global Outcomes (KDIGO) guidelines define CKD as abnormalities of kidney structure or function, present for more than 3 months, with implications to health (KDIGO, 2012). This definition has been formulated for the adult population, where CKD is a common and well-known health problem, but the KDIGO guidelines for definition and staging have many limitations when applied to the pediatric population, leading to markedly underestimate the burden of the problem and its long-term consequences (KDIGO, 2012). Indeed, pediatric CKD, while sharing the basic physio-pathologic mechanisms with the same disease in the adult population, could be in some ways considered a stand-alone nosologic entity that has only recently been recognized as a non-marginal issue (Becherucci F, 2016).

The first and more striking difference with the adult population is the etiology. Primary causes of CKD in children significantly differ from those that are responsible for the adult onset of the disease. In fact, the main etiologic factors of CKD in children are represented by congenital abnormalities of the kidney and urinary tract (CAKUT), steroid-resistant nephrotic syndrome (SRNS), chronic glomerulonephritis (e.g. lupus nephritis, Alport syndrome) and renal ciliopathies, that account for approximately 49.1, 10.4, 8.1 and 5.3% of cases,

respectively, representing more than 70% of all pediatric CKD cases when considered together (Vivante A, 2016) (Becherucci F, 2016). Less common causes of CKD in children include thrombotic microangiopathies (especially atypical hemolytic uremic syndrome, aHUS), nephrolithiasis/nephrocalcinosis, Wilms tumor, infectious and interstitial diseases, and others (Vivante A, 2016) (Becherucci F, 2016). While structural causes clearly predominate in younger patients, the incidence of glomerulonephritis increases in those older than 12 years (Vivante A, 2016) (Becherucci F, 2016). Among children who have already reached ESRD, the relative percentage of glomerular diseases increases (approximately doubling), whereas that of CAKUT decreases from around 50 to 39.5%, underscoring the discrepancy between the rates of progression of these two entities (Warady BA, 2007) (Becherucci F, 2016). The recent advent of next-generation sequencing (NGS) technologies has provided one of the most interesting and substantial clues in unraveling the etiology of early-onset CKD. Studies performed over the past few years have demonstrated that CKD manifesting before 25 years of age can be defined as monogenic in ~20% of cases (Vivante A, 2016). Nowadays, more than 200 genes are clearly recognized as causative of the most common etiologic categories of CKD in children (CAKUT, SRNS, chronic glomerulonephritis and ciliopathies) (Becherucci F, 2016) (Vivante A, 2016).

The long-term kidney outcomes of the broader spectrum of childhood kidney diseases remain largely unknown and difficult to define. Very recently, a nationwide, population-based historical cohort study among more than

1,000,000 adolescents with a long-term follow-up was conducted to evaluate whether a history of childhood kidney disease was associated with a risk of future development of ESRD (Calderon-Margalit R, 2018). This seminal study reported that a history of clinically evident kidney disease in childhood, even if renal function was apparently normal in adolescence, was associated with a significantly increased risk of ESRD, thus suggesting that kidney injury or structural abnormality in childhood have long-term consequences. This observation, together with the increase in the prevalence of CKD due to the improvement in patients survival, force us to remember that in the next future the number of affected adults facing problems that are specific to CKD that have started during childhood will strikingly increase and that recognizing kidney diseases occurring during childhood will be of fundamental importance in order to ensure patients with the most appropriate clinical and therapeutic management.

Genetics of inherited nephropathies: from monogenic diseases to risk alleles

Genetics first entered in the nephrology field in 1980s with the mapping of the genetic locus associated with autosomal recessive polycystic kidney disease (ARPKD) and with the identification of the first causative mutation responsible for a monogenic nephropathy, namely Alport syndrome. After these pivotal findings, a clear genetic cause has been recognized in many other inherited

kidney diseases, such as nephrogenic diabetes insipidus, Bartter and Gitelman syndrome, Dent disease, cystinosis and SRNS.

NGS (also referred to as massive-parallel or high-throughput sequencing) technology presents the striking advantage of simultaneously studying a high number of genes in a single run of sequencing, saving both time and costs while being extremely highly informative. Therefore, by selecting an appropriate panel of genes to sequence on the basis of the clinical phenotype of the patient or on a precise diagnostic suspicion, it is possible to address specific etiologic questions. The advent of NGS prompted to the identification of the molecular basis of more than 150 kidney disorders, whose majority could be defined as rare on the basis of epidemiological reports (Olivier Devuyst, 2014). Recent reports demonstrated that this could be the case also of early-onset CKD *per se* (Vivante A, 2016). These diseases are caused by mutations in gene encoding for proteins acting as cellular receptors, channels and transporters, enzymes, transcription factors and structural proteins, that can be expressed even in non-renal tissues (e.g. eye, inner ear, central nervous system), thus explaining the eventual presence of phenotypic extra-renal involvement in many disorders. These genes play a critical role for the development, survival and function of cells and tissues where they are expressed. Single mutations in these genes are responsible for the development of the disease (monogenic diseases), meaning that they are necessary and sufficient for determining the onset of the pathologic condition (large effect-size) (Hardies K, 2015).

Despite the huge improvement in the identification of the molecular basis of kidney diseases, the genetic cause is still lacking in many cases. Indeed, a clear genetic etiology can be identified in less than 50% of patients with SRNS, inherited tubulopathies or aHUS (Olivier Devuyst, 2014) (Vivante A, 2016). As a consequence, the use of genetic testing remains negligible even in these cases.

However, since their advent in late 1990s, NGS technologies (genome sequencing, whole-exome sequencing, target resequencing) have demonstrated efficiency, reliability e reproducibility, together with significant costs saving in comparison to traditional Sanger sequencing, thus challenging its role as gold standard for molecular diagnosis (Hardies K, 2015). Moreover, NGS have prompted sequencing studies in the clinical practice, even on a large scale, progressively shortening the distance "from the bench to the bedside".

The "NGS era" paved the way for a critical re-classification of many kidney diseases, including those currently considered as non-genetic (Olivier Devuyst, 2014). Beside this, NGS technology also raised questions that are still opened and far to be answered. They concern issues that are either intrinsic to the disease entity, like the pattern of inheritance (e.g. digenic inheritance), or related to the sequencing strategy, like the identification of large amount of variants of unknown clinical significance in each sequencing run. This represents one of the major challenges the "NGS era" launched to the medical community, including the nephrology one. Indeed, the definition of the pathogenic role of each variant needs the joint efforts of geneticists, nephrologists and bioinformatics in order to develop adequate disease

modeling and strategies for integration of clinical and genetic information (including reverse phenotyping) (Olivier Devuyst, 2014).

In addition, large population-based genetic studies (e.g. genome-wide association studies, GWAS) are revealing that the genetic background of kidney diseases is probably much more complex than what was previously expected. Indeed, besides a restricted number of clearly disease-causing genes, that are by themselves responsible for disease determination, a number of other genes are now recognized as playing an important role (Vivante A, 2016) (Gupta J, 2016). These variants have a high frequency and have been demonstrated to statistically associate with phenotypic traits, but with a small contribute to the determination of relative risk. This results in a low odds ratio (OR) for the disease (usually less than 1.5), making the cause-effect relationship difficult to demonstrate. Therefore, these variants are usually referred to as risk alleles, meaning that they could probably confer a risk of developing, but they are neither necessary nor sufficient for determining, the disease.

In the nephrology field, the best-known example is represented by *APOL1*, whose variants confer a considerably higher risk of developing focal segmental glomerulosclerosis (FSGS) and CKD progression (Gupta J, 2016) (Genovese G, 2010) (Tzur S, 2010) (Skorecki KL, 2013). The risk alleles of *APOL1*, called G1 and G2, have been demonstrated to be associated with an increased OR (7.3-29 times) and an earlier onset of ESRD and worse outcome after kidney transplant in African Americans (Freedman BI S. K., 2014). However, the principal role of

APOL1 risk alleles seems to be in favoring the progression of CKD (Skorecki KL, 2013) (Freedman BI & Consortium, 2014) (Freedman BI S. K., 2014).

Generally considered, these findings have substantial implications, either for the single patient or for more generalized considerations. First of all, patients with a recognized genetic cause of pediatric onset of CKD might benefit from specific therapies or from the avoidance of ineffective and even potentially health threatening ones (e.g. immunosuppressive drugs in patients with genetic forms of SRNS) (Giglio S, 2015) (Büscher AK B. B., 2014). In addition, molecular diagnostics enable prenatal testing in siblings of affected individuals and genetic counseling to the family, and may be of great help in assessing a patient prognosis. Finally, the categorization of disease entities by means of genetic testing is fundamental in assuring that the analysis of data from clinical research and pharmacologic trials is reliable.

Personalized medicine

The term "personalized medicine" is used widely in the media and in healthcare. However, what people refer to when they talk about personalized medicine can be variable (Ken Redekop W, 2013). Although subtle differences in the definition of what the personalized medicine is or should be, a general concept is that "personalized medicine is an emerging approach for disease treatment and prevention that takes into account individual variability in

genes, environment, and lifestyle for each person" (Health., 2018) (Ken Redekop W, 2013). The goal of personalized medicine is to determine the right drug, for the right patient, at the right time, tailoring and timing preventive as well as therapeutic measures (Gluba-Brzózka A, 2017). To this aim, the integration on biological information and biomarkers with genetics, proteomics as well as metabolomics is of fundamental importance. NGS is part of the techniques that are used to perform the analysis of genome and molecular interactions. Genetic findings should be implemented with the analysis of the related protein, resulting in a combined approach that should in the future provide real-time information of a person's physiological status, disease progression and outcome (Ken Redekop W, 2013). Moreover, the integration of genetic findings with pharmacological studies (pharmacogenetics and pharmacogenomics) will lead to the identification of novel potential therapeutic target to be specifically addressed in the single patient.

Moreover, personalized medicine (and nephrology) means the right diagnosis, the right prognosis prediction and the right genetic counseling to provide the patient and the family with. This means correct identification of the genetic cause of the disease, if present, understanding the role of the overall genetic background of each single patient and elucidation of potential genetic biomarkers of disease progression (e.g. rate of renal function deterioration leading to CKD and ESRD).

Whole-exome and whole-genome sequencing may revolutionize the approach to the disease and the patient resulting in the creation of "personalized

medicine" with new diagnostic and treatment strategies designed on the basis of genetic background of each individual (Gluba-Brzózka A, 2017).

Finally, since inherited nephropathies are mostly represented by rare disease, a personalized approach based on a deep knowledge of all the aforementioned issues, would probably lead to cost and time saving that will result in a rationale allocation of resources dedicated to healthcare and research (O'Donnell JC, 2013) and to additional benefit for all patients affected by rare kidney diseases.

Aim of the project

The aim of this project was to assess the potential application of NGS in the diagnosis of inherited nephropathies, with particular interest for SRNS and distal renal tubular acidosis (dRTA).

By a close correlation of genetic findings with deep phenotyping we aimed at:

1. Establishing the molecular basis of the disease;
2. Evaluating genotype-phenotype correlations;
3. Identifying factors influencing patients' prognosis;
4. Providing patients and families with adequate genetic counseling.

To this goal, we applied NGS strategies to a case of refractory lupus nephritis (LN), to a cohort of patients affected by dRTA and to a cohort of patients affected by sporadic SRNS.

Results

Next-generation sequencing and urine-derived renal progenitor cells for the diagnosis of refractory lupus nephritis

Response to treatment is one of the major determinants of the prognosis of many kidney diseases, including LN. Refractory lupus nephritis (RLN) is a term used to describe persistent or progressive renal dysfunction, no relevant decline in proteinuria within 3–6 months or no complete response at 24 months despite adequate treatment (Fanouriakis A, 2015). The pathogenic mechanisms of RLN are not fully understood.

In this work, we combined NGS and functional analysis of identified genetic variants in patient-derived podocytes to unravel the underlying cause of RLN in an adolescent.

Case report

A 14-years-old senegalese female was referred to the Nephrology unit because of fever, perimyocarditis, nephrotic-range proteinuria (8.5 g/day), hypocomplementaemia and antinuclear antibodies of 1:3200. In the clinical suspicion of LN, she underwent renal biopsy that confirmed the diagnosis, showing mesangial immune complex deposits with mild mesangial expansion, and no evidence of FSGS in 13 glomeruli. The patient responded well to

immunosuppressive (IS) therapy with steroids and mycophenolate mofetil (MMF) (Figure 1A) (Romagnani P G. S., 2016). One year later, a second biopsy showed absence of immune deposits but a single focal segmental sclerotic lesion in 1/19 glomeruli (Figure 1B), leading to a diagnosis of secondary FSGS. After 2 years of remission and cessation of IS treatment, the patient presented again with severe proteinuria (10 g/day). A third kidney biopsy showed class V LN and global sclerosis in 2/13 glomeruli (Figure 1B). Oral prednisolone, cyclosporine A and later MMF were started but nephrotic proteinuria persisted and a fourth biopsy showed class III/V (A/C) LN, 1/8 glomeruli with global sclerosis, interstitial fibrosis and tubular atrophy of 15–20% (Romagnani P G. S., 2016). RLN was addressed by rituximab and methylprednisolone treatment. This therapy controlled thrombocytopaenia and hypocomplementaemia but not nephrotic-range proteinuria (Figure 1A). Subsequently, CKD with hyperparathyroidism and hypertension became evident. To rule out persistently active LN a fifth biopsy was performed but showed class VI LN with global sclerosis in 3/3 glomeruli and 50% interstitial fibrosis and tubular atrophy (Figure 1B). Six months later the patient reached ESRD.

Genetic analysis with target resequencing and Sanger sequencing for *APOL1* risk alleles

In order to address previously unrecognized disease mechanisms of CKD progression that lead to the unfortunate outcome of this patient, we first performed genetic testing. As mentioned above, patients of African origin

carrying *APOL1* G1 and G2 risk alleles are at risk for CKD progression (Freedman BI & Consortium, 2014). This has been reported also for LN. Therefore, we performed Sanger DNA sequencing for *APOL1* gene. The patient resulted to be compound heterozygous for G1 and G2, one allele each inherited from her parents (Figure 2) (Romagnani P G. S., 2016). These findings could justify the rapid progression of the patient to ESRD, but was not sufficient to explain the lack of response to IS treatments. Indeed, in patients with *APOL1* risk alleles proteinuria usually responds to IS therapy (Kopp JB W. C., 2015), unlike children with steroid-resistant nephrotic syndrome, which in 30% a genetic podocytopathy can be identified by NGS (Giglio S, 2015). We therefore performed NGS target resequencing for a panel of genes responsible for inherited podocytopathies (Table 1), that has previously been demonstrated to allow us to recognized a genetic cause of the disease in 30% of children affected by SRNS (Giglio S, 2015) (Sadowski CE & SRNS Study Group, 2015). We identified the homozygous variant c.[1049C>T]+[1049C>T] in the *NPHS1* gene, inherited by each of the non-affected parents (Figure 2). This previously unreported variant results in the single amino-acid substitution of serine with phenylalanine in the position 350 of the protein nephrin.

Functional analysis of genetic variants in patient-specific podocytes obtained from urine-derived renal progenitor cells

Urine-derived renal progenitor cells (u-RPC) have been demonstrated to represent a useful and reliable tool for disease modeling of inherited

podocytopathies (Lazzeri E R. E., 2015).

To validate the pathogenicity of the genetic variants identified in the patient affected by RLN, we compared podocytes from the patient with those from controls by isolating $CD24^+/CD133^+$ u-RPC, expanding them in culture, and differentiating them into podocytes as previously described (Lazzeri E R. E., 2015). Phalloidin staining showed no difference between patient and controls before differentiation (Figure 3A, B) (Romagnani P G. S., 2016). However, after differentiation in podocytes, cells obtained from the patient showed an aberrant actin filament distribution in comparison with controls (Figure 3C, D, C0, D0) and were less viable upon differentiation, as assessed by flow cytometry analysis of the spontaneous uptake of Annexin V/Propidium Iodide (Figure 3E, F). Since *APOL1* risk variants can also enhance podocyte death *via* lysosomal destabilization, we perform lysosomal staining with the lysosomal marker Lysotracker in podocytes obtained from the patients and from the controls. Consistently, the patient's podocytes displayed a significantly lower staining with Lysotracker in comparison with controls as a sign of lysosomal destabilization (Figure 3G–I). We then examined the expression of the protein nephrin. Although nephrin mRNA levels were similar in the patient and controls (data not shown), confocal microscopy for nephrin protein revealed nephrin properly localizing to the outer plasma membrane in control podocytes (Figure 3J, J0), while the patient's podocytes showed significant aberrant nephrin localization within the cytoplasm (Figure 3K, K0).

Discussion

Starting from the observation that lack of response to treatment represents one of the most important determinants of CKD progression, independently on the cause of CKD, we hypothesized that the entity of RLN may also include patients with genetic lupus podocytopathies (Bomback AS, 2016). NGS in our patient showed the presence of a homozygous variant in the *NPHS1* gene, one allele each inherited from each of her healthy heterozygous parents, in line with the autosomal-recessive transmission of nephrotic syndrome of the Finnish type. A point mutation in the same position causing substitution of serine with proline was reported in a compound heterozygous status in a case with congenital nephrotic syndrome and is listed in Human Gene Mutation Database (Lenkkeri U, 1999) (Liu L, 2001). This amino acid is highly conserved and its replacement by phenylalanine is predicted as potentially damaging by *in silic*o analysis. This disease most commonly presents as congenital nephrotic syndrome, but cases with a juvenile onset have been reported, depending on the genomic impact on protein amount, shape and location (Santín S & Group, 2009). A careful structural and functional characterization of podocytes derived from u-RPC is a powerful diagnostic tool for modeling genetic podocytopathies (Lazzeri E R. E., 2015). In our patient, podocytes derived from u-RPC showed disturbed nephrin trafficking to the cell surface, an abnormal cytoskeletal structure, and reduced cell viability similar to what has been previously reported in patients with nephrotic syndrome of the Finnish type (Liu L, 2001). These findings validate the pathogenicity of the patient's *NPSH1* gene variant

and provide a possible explanation for: (i) accelerated podocyte loss and glomerulosclerosis, (ii) the delayed onset of nephrotic syndrome and (iii) the multidrug resistance of the patient (Romagnani P G. S., 2016). In addition, the patients carried the G1 and G2 risk alleles in *APOL1* gene. This genetic finding is consistent with a high risk for an accelerated progression of CKD to ESRD. The results of this study suggest that one of the mechanisms responsible for this clinical behavior could be represented by accelerated podocyte loss *via* lysosomal leakage-driven podocyte detachment and death (Lan X, 2014).

Taken together, the results of this study underline the need for genetic screening in RLN. Early diagnosis of genetic disease is important for prognosis prediction, to avoid unnecessary immunosuppressive therapy and for genetic counseling of family members.

Figures and Tables

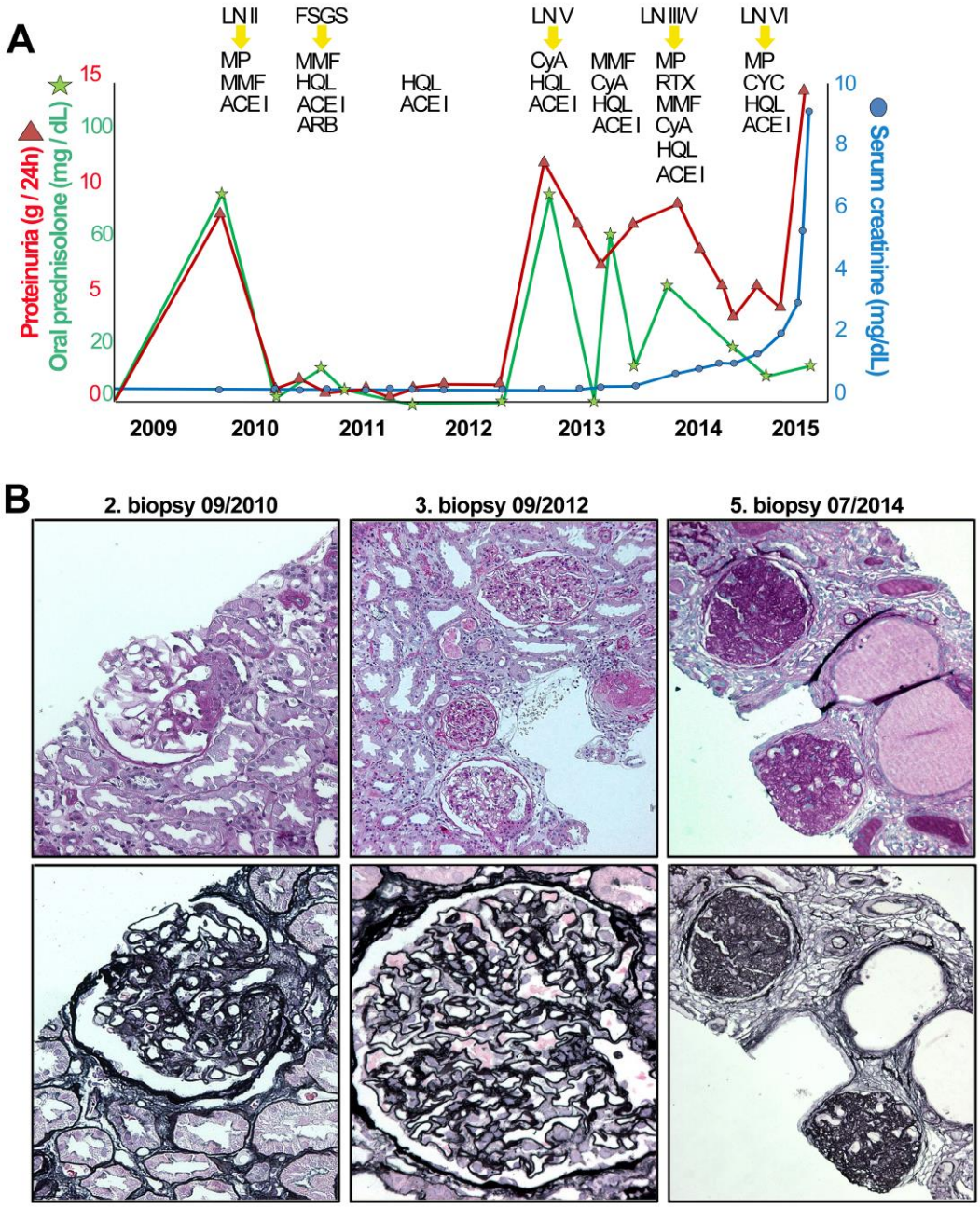

Figure 1. Clinical course of RLN and kidney biopsy results

(A) The graph illustrates proteinuria as assessed by 24 hours urine collection (red), serum creatinine levels (blue) and the dose of oral prednisolone (green).

Yellow arrows indicate kidney biopsies, whose main results are listed above. The consecutive treatment regime is indicated below. (B) From the five kidney biopsies available for this patient, the 2nd, 3rd and 5th are. PAS and silver staining are shown at a magnification of 200×. Assessment was performed according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification of LN. Left: FSGS in the absence of proliferative immune complex disease. Middle: ISN/RPS class V with 15% interstitial fibrosis and tubular atrophy. Right: ISN/RPS class VI with >50% interstitial fibrosis and atrophy.

LN, lupus nephritis; FSGS, focal segmental glomerulosclerosis; MP, intravenous methylprednisolone; MMF, oral mycophenolate mofetil; ACEI, oral angiotensin-converting enzyme inhibitor; HQL, hydroxychloroquine; ARB, angiotensin receptor blocker; CyA, cyclosporine A; RTX, rituximab; CYC, cyclophosphamide.

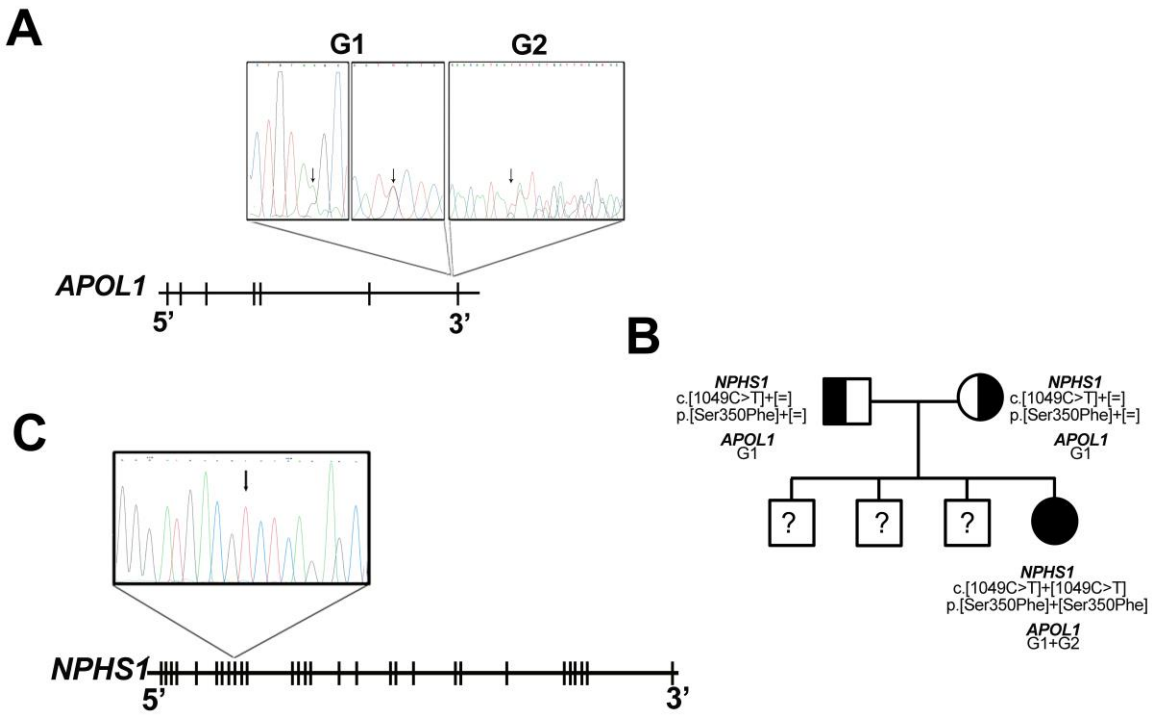

Figure 2. Sequencing of *APOL1* and podocyte genes.

(A) Schematic structure of the human *APOL1* gene showing the risk alleles G1 and G2 identified in the patient affected by LN. Exons are indicated as rectangles. (B) The family pedigree of the patient displays the recessive pattern of inheritance of the risk alleles G1 and G2 in *APOL1* gene. The same pattern of inheritance is presented by variants in *NPHS1* gene. (C) Schematic structure of the human *NPHS1* gene showing the variant c.[1049C>T]+[1049C>T] identified in the patient affected by LN by NGS. Exons are indicated as rectangles.

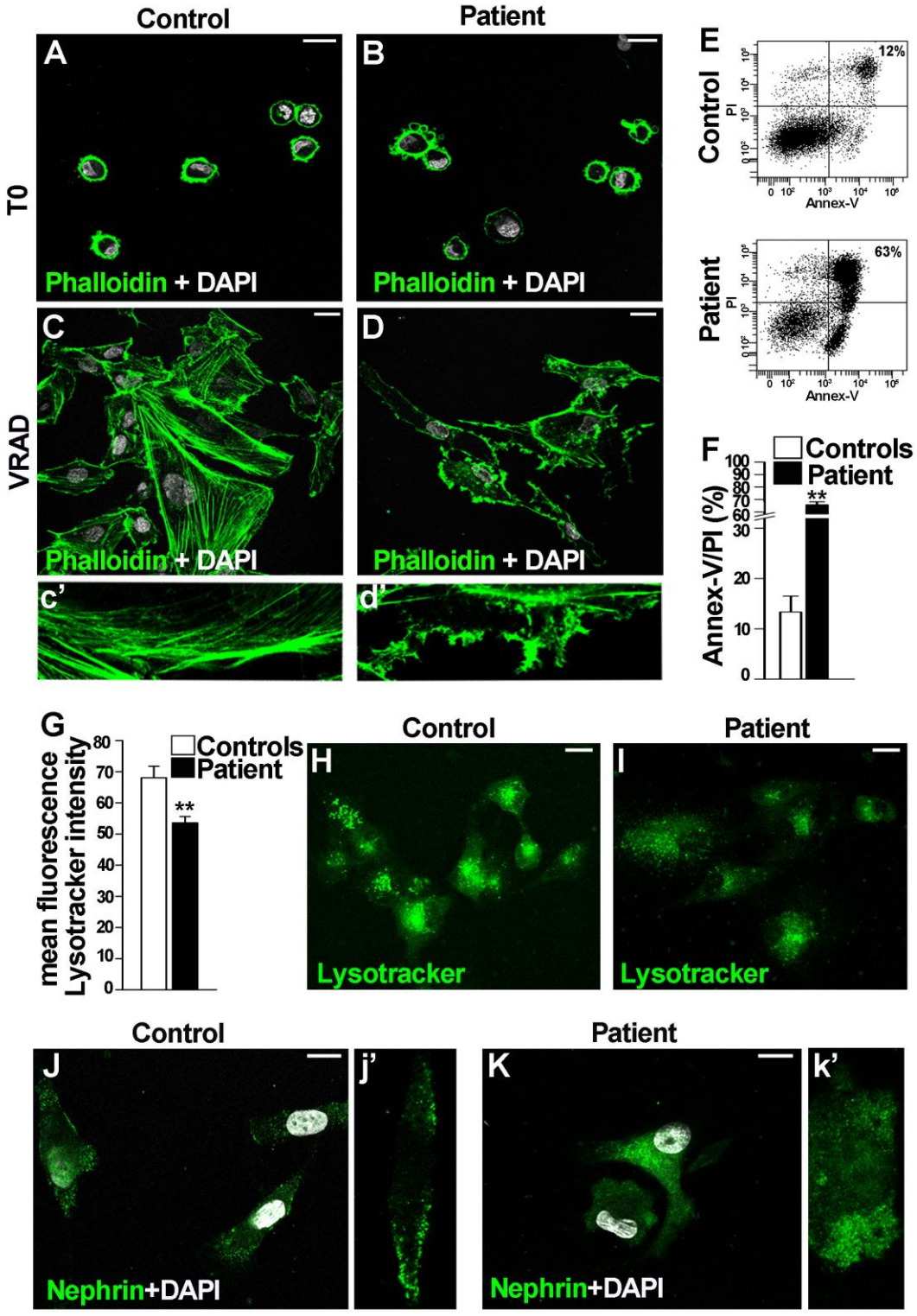
Control
Patient
T0
VRAD
A
B
C
D
c'
d'
Phalloidin + DAPI
E
Control
Patient
12%
63%
PI
Annex-V
F
Controls
Patient
Annex-V/PI (%)
**
G
mean fluorescence Lysotracker intensity
H
I
Lysotracker
J
K
j'
k'
Nephrin+DAPI

Figure 3. Functional characterization of urine-derived renal progenitor cells and podocytes from the patient and controls.

(A–D) Distribution of actin filaments by Phalloidin staining in u-RPC (A, B) and podocytes (C, C0, D, D0) is shown in green. Altered cytoskeleton architecture of podocytes obtained from u-RPCs of the patient affected by LN carrying the risk alleles G1/G2 in *APOL1* gene and the genetic variants in *NPHS1* gene (D, D0) in comparison with podocytes obtained from a control (C, C0). DAPI counterstains nuclei (white). Bars = 20 μm. Data from one out of five controls are shown. One representative out of four independent experiments is shown. (E) Percentage of dead cells following differentiation into podocytes of u-RPC obtained from controls (above) and from the patient carrying the risk alleles G1/G2 in *APOL1* gene and the genetic variants in *NPHS1* gene (below), as assessed by spontaneous uptake of Propidium Iodide (PI) and Annexin V (Annex-V) by flow cytometry analysis. Data from one out of five controls are shown. One representative out of four independent experiments is shown. (F) Graph representing percentage of Annex-V/PI-positive cells in controls (white column) and in the patient (black column), obtained in at least four separate experiments. Data are shown as mean ± SEM. **P < 0.001 by Mann–Whitney Test. (G) Graph representing mean ± SEM of fluorescence intensity of Lysotracker dye in controls (white column) and in the patient (black column). The mean fluorescence intensity was measured per region of interest. One representative out of four independent experiments is shown. **P < 0.001 by Mann–Whitney test. (H-I) Assessment of lysosomal structural integrity by

Lysotracker dye (green) in the control (H) and the patient (I). DAPI counterstains nuclei (white). Bars = 20 μm. Data from one out of five controls are shown. One representative out of four independent experiments is shown. (J, J0, K, K0) Expression of nephrin (green) after differentiation into podocytes of u-RPC obtained from control (J, J0) and the patient carrying genetic variants in *NPHS1* gene (K, K0). DAPI counterstains nuclei (white). Bars = 20 μm. Data from one out of five controls are shown. One representative out of four independent experiments is shown.

Table 1. List of genes responsible of glomerular diseases included in the NGS sequencing array.

Gene	Proteina	Locus	Esone	RefSeq
WT1	Wilms tumor	chr11:32409325-32457087	10	NM_024426
LMX1B	LIM homeobox transcription factor 1, beta	chr9:129376748-129463311	8	NM_002316
NPHS1	nephrin	chr19:36316274-36342739	29	NM_004646
NPHS2	podocin	chr1:179519677-179545084	8	NM_014625
CD2AP	CD2-associated protein	chr6:47445525-47594994	18	NM_012120
ACTN4	actinin, alpha 4	chr19:39138327-39221170	21	NM_004924
INF2	inverted formin 2	chr14:105155943-105185947	23	NM_022489
ITGA3	integrin alpha 3	chr17:48133340-48167848	26	NM_002204
TRPC6	transient receptor potential cation channel,6	chr11:101322296-101454659	13	NM_004621
LAMB2	laminin, beta 2	chr3:49158548-49170599	33	NM_002292
PLCE1	phospholipase C, epsilon 1	chr10:95753746-96088146	32	NM_016341
SCARB2	scavenger receptor class B, member 2	chr4:77079894-77135035	12	NM_005506
CoQ2	para-hydroxybenzoate-polyprenyltransferase,	chr4:84184979-84205964	7	NM_015697
PDSS2	prenyl diphosphate synthase, subunit 2	chr6:107473761-107780779	8	NM_020381
SMARCAL1	SWI/SNF-related matrix-associated	chr2:217277473-217347772	18	NM_001127207
ZMPSTE24	zinc metallo-peptidase STE24	chr1:40723733-40759855	10	NM_005857
MYH9	myosin, heavy	chr22:36677324-36784063	42	NM_002473

	polypeptide 9, non-muscle			
PTPRO	receptor-type protein tyrosine phosphatase O	chr12:15475487-15750335	27	NM_030667
MYO1E	myosin IE	chr15:59428564-59665071	28	NM_004998
PAX2	paired box 2	chr10:102505468-02589697	11	NM_003990
ARHGDIA	rho GDP dissociation inhibitor (GDI) alpha	chr17:79825597-9829282	6	NM_004309
ARHGAP24	rho GTPase activating protein 24	chr4:86396284-86923823	10	NM_001025616
CUBN	Cubilin	chr10:16865965-17171816	67	NM_001081
ANLN	anillin actin binding protein	chr7:36429432-36493400	24	NM_018685
TTC21B	tetratricopeptide repeat domain 21B	chr2:166729872-166810348	29	NM_024753
COL4A3	collagen type IV alpha 3	chr2:228029281-228179508	52	NM_000091
COL4A4	collagen type IV alpha 4	chr2:227867427-228029275	48	NM_000092
COL4A5	collagen type IV alpha 5	chrX:107683074-107940775	53	NM_033380
COL4A6	collagen type IV alpha 6	chrX:107398837-107681658	45	NM_033641

Phenotypic and genetic characterization of patients affected by distal renal tubular acidosis

Distal renal tubular acidosis (dRTA) is a rare genetic disorder usually presenting with unspecific clinical symptoms such as vomiting, diarrhea and/or constipation, loss of appetite, polydipsia and polyuria, nephrocalcinosis, nephrolithiasis, osteomalacia, and rickets, and growth retardation. Laboratory tests show non-anion gap metabolic acidosis and inability to maximally acidify the urine due to an alteration in the a-intercalated cells in the collecting duct (Rodríguez Soriano J, 2002). dRTA can be transmitted either as an autosomal dominant (AD) or autosomal recessive (AR) trait (Pereira PC, 2009). AD forms typically become clinically manifest in adolescence or adulthood and are usually caused by mutations in the *SLC4A1* gene, encoding the basolateral $Cl^-/HCO3^-$ exchanger (Rodríguez Soriano J, 2002) (Bruce LJ, 1997). Due to alternative splicing in the kidney and in erythrocytes, this gene could be responsible for dRTA and/or hemolytic anemia with red cell morphology anomalies, also in a recessive manner (Tanphaichitr VS, 1998). On the other hand, AR dRTA is associated with mutations in *ATP6V0A4* and *ATP6V1B1* genes encoding for the A4 and B1 subunits of the apical H^+ATPase pump, respectively. Early-onset sensorineural hearing loss (SNHL) can be an accompanying clinical feature of AR dRTA. As reported in the current literature, in these cases the disease is usually caused by mutations in the *ATP6V1B1* gene (Karet FE, 1992).

The diagnosis of dRTA is based on clinical and laboratory features. However, a molecular diagnosis is of great importance in order to define genotype-phenotype correlations and to provide patients with a personalized clinical management. Since the disease is extremely rare, only small series of patients with dRTA has been studied and genetically characterized so far, making strict and reliable genotype-phenotype correlations is still lacking.

In this part of the project, we applied a NGS to a cohort of 89 patients with a clinical diagnosis of dRTA referred to Meyer Children's Hospital of Florence for clinical evaluation or molecular diagnosis.

Genetic analysis with target resequencing

All the 89 patients underwent genetic testing with NGS for *SLC4A1*, *ATP6V1B1*, and *ATP6V0A4* genes. Among the entire cohort, 64 showed causative mutations (71.9%) in 1 of the 3 genes (Figure 4a) (Palazzo V, 2017): 9 patients showed pathogenic variants in the *SLC4A1* gene (10.1%), 30 patients in the *ATP6V0A4* gene (33.7%), and 25 patients in the *ATP6V1B1* gene (28.0%) Among patients carrying pathogenic variants in the *SLC4A1* gene, 7 (6.7%) satisfied criteria for a molecular diagnosis of AD dRTA, whereas 2 (2.2%) were homozygous. All the mutations were missense, and 6 of them were *de novo*. All of these variants were already reported as pathogenic (Palazzo V, 2017). We fund a high frequency of compound heterozigousity either in the *ATP6V0A4* gene, or in the *ATP6V1B1* gene, as expected in non-inbred populations. Moreover, 7 additional patients (7.9%) were found to have genetic variants that did not satisfy criteria for a

conclusive molecular diagnosis and were therefore classified as variants of unknown clinical significance (Figure 4a). In particular, 5 patients presented heterozygous variants in the *ATP6V0A4* gene or the *ATP6V1B1* gene, whereas 2 cases presented 2 different variants in a "digenic" pattern of inheritance, involving the *ATP6V0A4*/*ATP6V1B1* genes in one case and the *ATP6V1B1*/*SLC4A1* genes in another case (Palazzo V, 2017). Interestingly, all of these patients presented with the classic clinical features of dRTA. In addition, in the completely negative patients (18 patients) as well as in all of the *ATP6V0A4* and *ATP6V1B1* heterozygous cases (5 patients), the a-CGH allowed us to exclude any imbalance rearrangements inside the genes or in their flanking regions.

Genotype-phenotype correlation

In the majority of patients included in this study, the clinical suspicion of dRTA arose because of failure to thrive (FTT) and vomiting, and the clinical diagnosis was confirmed with laboratory tests showing hypercloremic metabolic acidosis with a simultaneous positive urinary anion gap and the inability to maximally acidify the urine. Biochemical data resulting from provocative tests were not available in our cohort. Although no significant difference was evident by analyzing the mean values of venous blood pH and urinary pH (Figure 5a-b) in patients with and without pathogenic mutations, levels of serum $HCO3^-$ and potassium were slightly different (Figure 5c-d, Student *t* test, $P < 0.05$), suggesting a more severe grade of metabolic acidosis in patients with

mutations that is not fully reflected only by the mean of blood pH. The male/female ratio of the subjects included in the study was 43/46 (48.3% male) (Table 2). The great majority of patients were from unrelated families of Caucasian origin, and more than 70% of cases were sporadic.

Patients with causative mutations showed a mean age at clinical diagnosis of dRTA of 65.2 months in the group of (Table 2, Figure 4b). Consistent with previous reports, the mean age at diagnosis was significantly older in patients with mutations in the *SLC4A1* gene (153.2 months; range, 6–540 months) compared with that of patients with mutations in the *ATP6V1B1* gene (13.9 months; range, 1–60 months; Mann-Whitney U test=211.5, $P < 0.001$) or in the *ATP6V0A4* gene (28.6 months; range, 1–360 months; Mann-Whitney U test=243, $P < 0.001$). Patients without mutations had a mean age at clinical diagnosis of 131.1 months (range, 1–600 months) (Table 2, Figure 4b). The age at diagnosis was significantly different in the group of patients with mutations compared with those without mutations (Mann-Whitney U test=181.5, $P < 0.05$) (Figure 4b). On the contrary, no significant difference was found by comparing the group with mutations and the group of patients carrying variants of unknown clinical significance (Mann-Whitney U test=166, not significant) (Figure 4b).

Surprisingly, 1 case of SNHL was reported among patients with pathogenic variants in the *SLC4A1* gene (Table 2, Figure 4c). In addition, 92% were reported to have hearing impairment in the group of patients carrying causative mutations in the *ATP6V1B1* gene. Moreover, 17 of 30 patients with pathogenic variants in the *ATP6V0A4* gene had SNHL, determining a

prevalence of such a phenotypic feature as high as 56.7% (Table 2, Figure 4c). This frequency, although significantly lower than that found in patients with causative mutations in the *ATP6V1B1* gene (chi-squared=6.89, $P < 0.05$), was slightly higher than that reported in patients with mutations in the *ATP6V0A4* gene described so far (Smith AN, 2000) (Miura K, 2013). In patients with mutations in the *ATP6V1B1* gene, SNHL has an earlier clinical onset compared with patients carrying mutations in the *ATP6V0A4* gene (Mann-Whitney U test=93, $P < 0.05$) (Table 2, Figure 4d). Interestingly enough, however, patients with mutations in the *ATP6V0A4* gene presented a wider range of clinical onset of SNHL (range, 2–552 months) (Figure 4d), encompassing infancy and early childhood, thus suggesting that an early onset of this clinical feature is not exclusive of *ATP6V1B1* gene variants. In addition, SNHL was found in 3 of 18 patients (16.7%) negative at the genetic testing for the 3 genes (Table 2, Figure 4c); in this group, the age at onset was particularly variable (Table 2, Figure 4d). On the other hand, in patients carrying variants of unknown clinical significance, 42.9% had SNHL, a frequency not significantly different from that found in patients with mutations (chi-squared=0.55, not significant).

The frequency of nephrocalcinosis, FTT and hypokaliemia was not significantly different in the three groups of patients with mutations (Table 2, Figure 4e-g). All these features were present at a high frequency in patients with variants of unknown clinical significance (Table 2, Figure 4e-g). Interestingly enough, patients with mutations in genes encoding the A4 or B1 subunit of the H^+ATPase pump had a more severe hypokalemia compared with those with

mutations in the *SLC4A1* gene, consistent with previous findings (Table 2) (Batlle D, 2006). In any case, no clinical phenotype attributable to hypokalemia was reported in the clinical records. CKD was present in 31.3% of patients with pathogenic mutations (Table 2). This frequency, although not significantly different compared with that found in patients with negative results on the genetic screening (chi-squared=0, not significant) or in patients with mutations of unknown clinical significance (chi-squared=0.49, not significant) is higher than that reported so far (Haffner D, 1999) (Rodríguez Soriano J, 2002). Furthermore, CKD was evident in patients with long-term follow-up (at least 15 years) and always after puberty (Figure 6).

Discussion

In this study, we described a large cohort of patients with dRTA, finding a close correlation between the clinical phenotype and the results of genetic testing (Palazzo V, 2017). This approach allowed us to make some unexpected observations with important implications for the clinical management of patients affected by this disease: (i) most cases of dRTA are "sporadic" (>70%), although genetically transmitted, deriving from homozygous or compound heterozygous mutations; (ii) mutations in the *ATP6V0A4* gene are quite as frequent as mutations in the *ATP6V1B1* gene in patients with AR dRTA; (iii) in contrast to previous observations, the association of dRTA with early SNHL is not an absolute indicator of the underlying causal gene; (iv) CKD is more

frequent than reported thus far and can occur in patients with a long history of the disease.

Most studies published to date described affected individuals grouped within families. In this study, we showed that the large majority of cases occur in unrelated families, suggesting that a genetic form of dRTA should be suspected even in the absence of a familial history of the disease or consanguinity. Consistently, the cases reported in this study showed causative mutations transmitted in compound heterozygousity.

By analyzing the available clinical, laboratory, and instrumental data of 89 patients suspected of having dRTA, we can conclude that SNHL, nephrocalcinosis, FTT, and age at diagnosis actually allow patients with pathogenic mutations in 1 of the 3 causative genes to be differentiated from patients who are negative, thus suggesting that the initial clinical diagnosis of dRTA in the latter group should be reconsidered. Indeed, negative patients showed a less severe degree of metabolic acidosis and hypokalemia that, although clinically negligible, could be suggestive of a different pathogenesis. On the other hand, patients carrying variants of unknown clinical significance were not statistically different from those with pathogenic mutations for the majority of the clinical parameters, indicating that the variants identified in the first group are probably not insignificant in determining the clinical phenotype of patients. Anyway, the clinical significance of these genetic findings remains to be clarified and it is reasonable to hypothesize also that other genes expressed in the distal tubule or in other tubular segments and still unidentified

could be mutated. Similarly, in patients who have a clinical phenotype consistent with dRTA and who were negative for the 3 known causative genes, it is possible that other genes potentially expressed in other tubule regions mimic the same clinical picture.

From a clinical perspective, the only parameters that presented a statistically significant difference in the 3 groups of mutated patients were, not surprisingly, the age at onset and the presence of SNHL that allowed us to distinguish patients with mutations in the *SLC4A1* gene from those with mutations in the *ATP6V1B1* or *ATP6V0A4* genes. However, although patients with mutations in the *ATP6V1B1* gene had a higher prevalence and an earlier clinical onset of SNHL compared with patients carrying pathogenic variants of *ATP6V0A4*, SHNL should perhaps not be considered as an exclusive feature of the former group of patients. Our data suggest that these 2 genes must both be analyzed when dRTA is suspected, especially if SNHL is present and the patient is young.

Finally, we found a surprisingly high frequency of CKD in patients carrying pathogenic mutations. This is in contrast to what is reported in the literature (Haffner D, 1999) (Rodríguez Soriano J, 2002). CKD occurs in patients with a long history of the disease and could be explained by the combination of nephrocalcinosis and persistent hypokalemia, leading to progressive tubulo-interstitial damage, or by kidney damage following repeated episodes of dehydration and acute kidney injury. Interestingly enough, in our patients, CKD never occurred before adolescence and a pubertal growth spurt, probably

because of compensatory hyperfiltration of functioning nephrons during childhood. This observation deserves particular attention because it suggests that, in the absence of previous clinical records, the diagnosis of dRTA could be missed in young adults presenting with moderately elevated serum creatinine levels and metabolic acidosis, as previously anecdotally reported (Gee HY O. E., 2014). Moreover, it raises the question on the "benignity" of the disease. Obviously, these data need to be confirmed in larger cohorts of patients with dRTA.

Taken together, these results allow us to conclude that in the case of clinically suspected dRTA, the presence of accompanying clinical and laboratory features such as SNHL, nephrocalcinosis, and FTT and a less severe degree of metabolic acidosis and hypokalemia suggests that genetic testing should be performed. This testing should always include the analysis of all 3 genes performed by NGS. In addition, genetic testing could help in predicting the long-term prognosis of patients with dRTA, helping clinicians in the overall management of the disease, i.e. avoiding the exposure to additional offending factors leading to CKD progression (e.g. nephrotoxic drugs, dehydration).

Figures and Tables

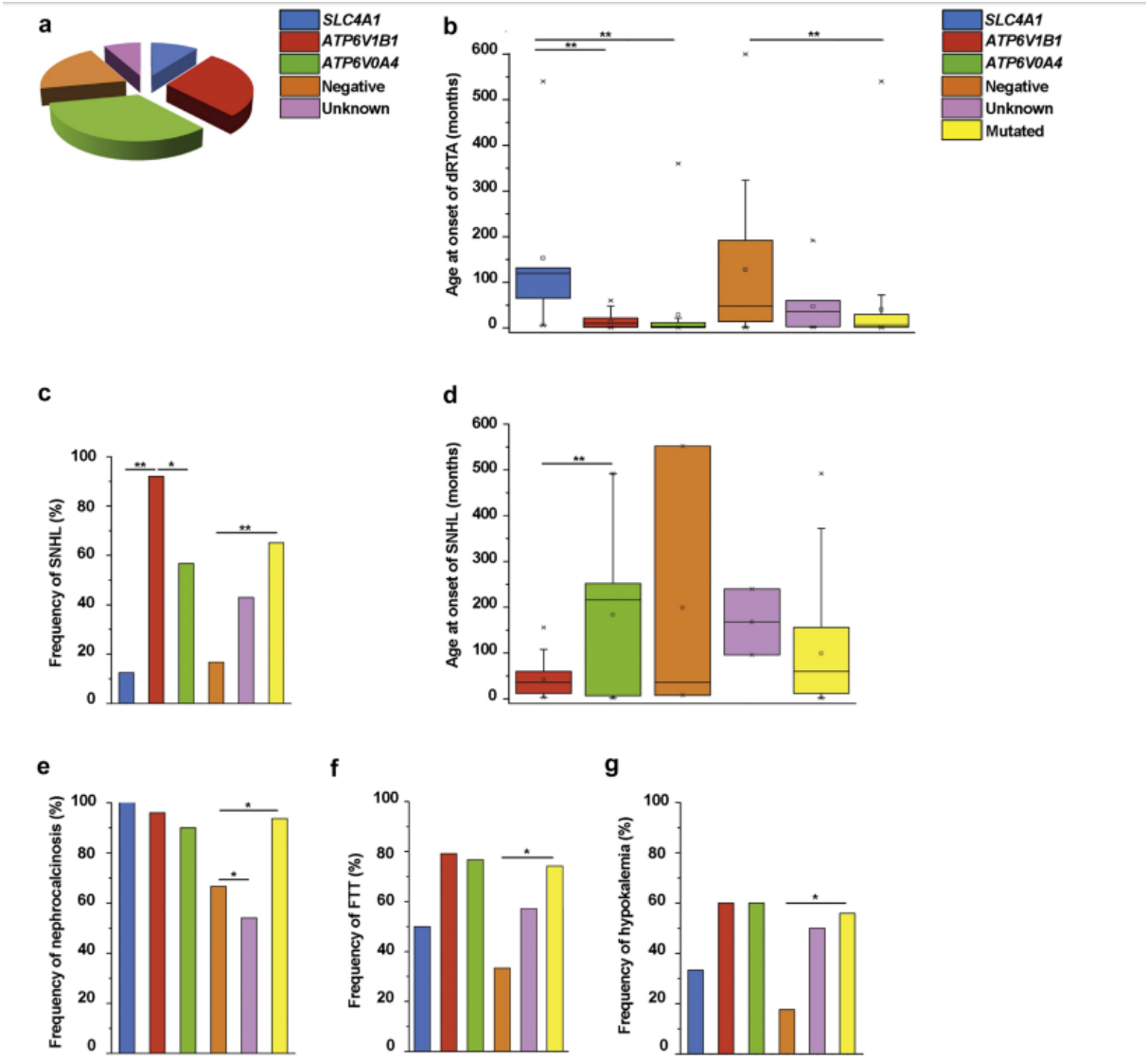

Figure 4. Molecular characterization and clinical features of patients with distal renal tubular acidosis.

(a) Results of the genetic testing performed in 89 patients with a clinical diagnosis of dRTA. The frequency of mutations in *SLC4A1*, *ATP6V1B1*, and *ATP6V0A4* genes responsible for dRTA is reported. The frequency of negative cases and of variants of unknown clinical significance is also reported. (b) Age at clinical diagnosis. (c) Frequency of SNHL. (d) Age at onset of SNHL. (e)

Frequency of nephrocalcinosis. (f) Frequency of failure to thrive (FTT). (g) Frequency of hypokalemia.

Throughout the figure, blue represents patients with pathogenic mutations in the *SLC4A1* gene (either autosomal dominant or autosomal recessive), red represents patients with pathogenic mutations in the *ATP6V1B1* gene (AR), green represents patients with pathogenic mutations in the *ATP6V0A4* gene (AR), orange represents patients without pathogenic mutations in the 3 genes analyzed, lilac represents patient carrying mutations that did not satisfy criteria for a molecular diagnosis (heterozygous variants in *ATP6V1B1* or *ATP6V0A4* genes or digenic inheritance; unknown), and yellow represents patients with pathogenic mutations in 1 of the 3 analyzed genes (mutated=*SLC4A1*+ *ATP6V1B1*+*ATP6V0A4*). *P < 0.05; **P < 0.001.

In each box-and-whiskers graph, the bottom and top of the box are always the first and third quartiles, and the band inside the box is always the second quartile (the median). Squares inside each box represent the mean value for each group. The whiskers indicate variability outside the upper and lower quartiles, with the end of the whiskers representing 5th and 95th percentiles.

SNHL, sensorineural hearing loss; FTT, failure to thrive.

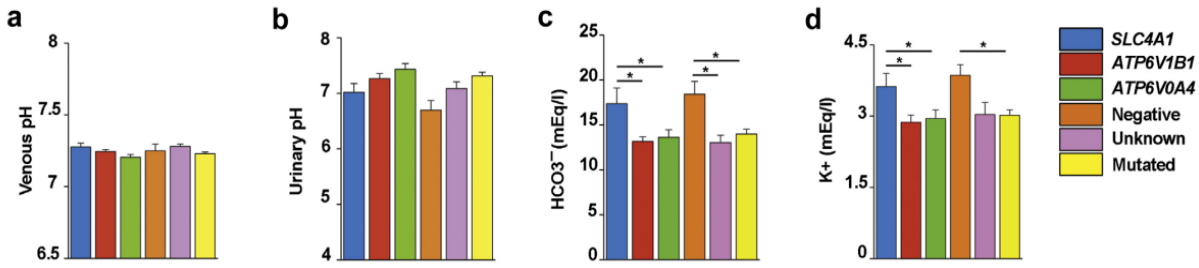

Figure 5. Summary of data for laboratory findings.

Venous blood pH. (b) Urinary pH. (c) Serum $HCO3^-$ (mEq/l). (d) Serum potassium (mEq/l). Serum creatinine levels at onset (mg/dl). Serum creatinine levels at last follow-up (mg/dl). *P < 0.05.

The same color code described above is applied.

HCO3-, serume bicarbonate; K^+, serum potassium.

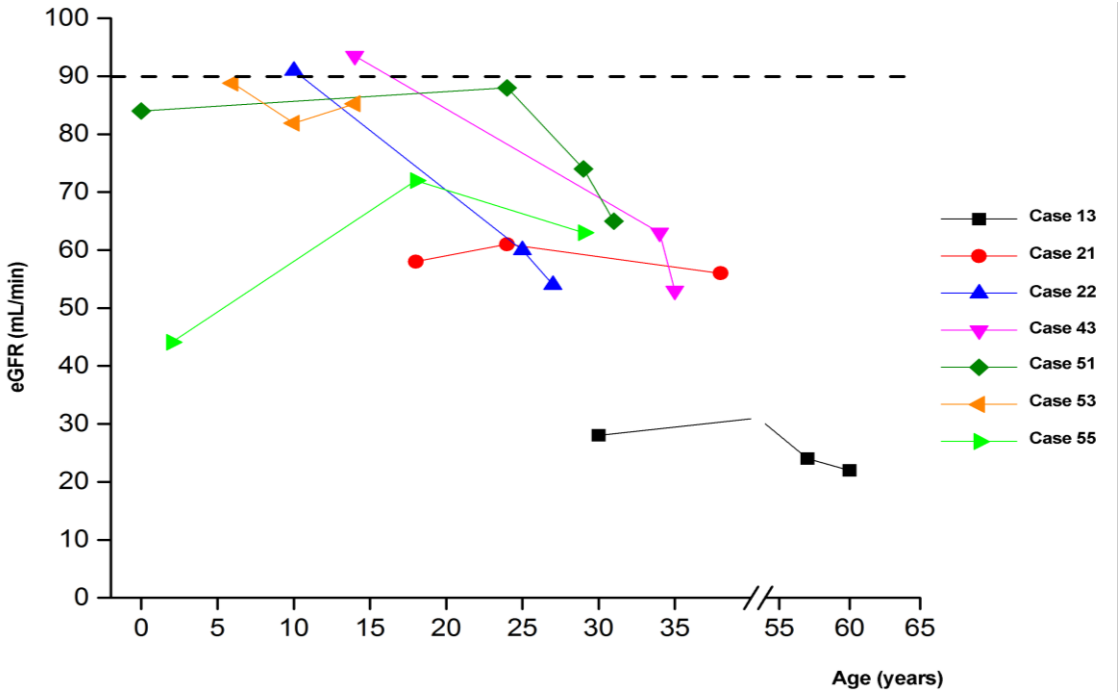

Figure 6. Plot of the estimated glomerular filtration rate over time in a subset of patients with pathogenic mutations.

Schematic representation of CKD progression by plotting the estimated glomerular filtration rate over time in a subset of patients with progressive worsening of renal function and pathogenic mutations in whom at least 3 measurements of serum creatinine were available.

eGFR, estimated glomerular filtration rate.

Table 2. Summary of clinical features of patients included in the study.

	SLC4A1	*ATP6V1B1*	*ATP6V0A4*	Variants of unknown clinical significance	Negative	Mutated
M/F, no. (%)	4/9 (44.4)	13/25 (52)	14/30 (46.6)	5/7 (71.4)	7/18 (38.9)	31/64 (48.4)
Age at onset of dRTA, mo	153.2	13.9	28.6	47.6	131.1	65.2
SNHL, no. (%)	1/8 (12.5)	23/25 (92)	17/30 (56.7)	3/7 (42.9)	3/18 (16.7)	41/63 (65)
Age at onset of SNHL, mo	240	41.8	183.5	168	198.7	155.1
Nephrocalcinosis, no. (%)	8/8 (100)	24/25 (96)	27/30 (90)	4/7 (57.1)	12/18 (66.6)	59/63 (93.6)
FTT, no. (%)	4/8(50)	19/24 (79.1)	23/30 (76.6)	5/6 (83.3)	2/21 (9.5)	46/62(74.2)
Hypokalemia, no. (%)	3/9 (33.3)	15/25 (60)	15/25 (60)	3/6(50)	3/17(17.6)	33/59 (55.9)
CKD		16/51 (31.3)		2/7 (28.6)	5/14 (35.7)	16/51 (31.3)

CKD, chronic kidney disease dRTA, distal renal tubular acidosis; FTT, failure to thrive; M/F, male/ female; SNHL, sensorineural hearing loss.

Whole-exome sequencing for personalized diagnosis of steroid-resistant nephrotic syndrome in young patients

Primary idiopathic nephrotic syndrome (NS) is classified according to the response to steroids in steroid-sensitive (SSNS) or SRNS (Glomerulonephritis Work Group: Improving Global Outcomes (KDIGO), 2012). While SSNS usually has a favorable prognosis, SRNS can either undergo remission or progress to ESRD (Tarshish P, 1997) (Gipson DS, 2006) (Büscher AK B. B., 2014) (Trautmann A S. S.-Z., 2017). Genetic testing has become a valuable diagnostic tool to identify a monogenic podocytopathy in about 30% of cases with SRNS (Büscher AK B. B., 2014) (Giglio S, 2015) (Sadowski CE & SRNS Study Group, 2015) (Wang F Z. Y., 2017) (Bierzynska A, 2017) (Trautmann A B. M., 2015). Patients with podocytopathy-related SRNS typically show FSGS and are frequently resistant not only to steroids, but also to other IS drugs. (Büscher AK B. B., 2014) (Giglio S, 2015) (Trautmann A S. S.-Z., 2017) (Büscher AK K. B., 2012). The remaining 70% of SRNS patients are currently considered to be of non-genetic cause, but despite some respond to IS drugs in terms of proteinuria reduction, many are still multidrug-resistant and anyway progress to ESRD (Büscher AK K. B., 2012) (Trautmann A S. S.-Z., 2017). SRNS and FSGS can sometimes also be secondary to other genetic nephropathies, where they associate with other clinical features that make the underlying disease easily recognizable. However, mitochondriopathies, Alport syndrome, Dent or Fabry disease, cystinosis, presenting with SRNS and FSGS in apparent absence of other symptoms, i.e. primary SRNS phenocopies, have been occasionally reported (Malone AF, 2014)

(Vallés P, 2000) (van Berkel Y, 2017) (Wang X, 2016) (Koulousios K, 2017) (Köping M, 2017) (Chandra M, 2010) (Bonsib SM, 1999) (Dhooria GS, 2014) (Servais A, 2008). A phenocopy is a phenotypic trait or disease that resembles the trait expressed by a particular genotype, but in an individual who is not carrier of that genotype. Indeed, when specific signs of the underlying disease are even absent in other family members, a clinical diagnosis without genetic testing is virtually impossible (Sampson MG, 2015). We hypothesized that such cases may explain the multidrug resistance in at least some young sporadic patients with SRNS. To address this concept, we used an unbiased approach by employing whole-exome sequencing (WES) followed by bioinformatic filtering of all genes previously related to kidney diseases in young patients with a clinical diagnosis of idiopathic NS followed by reverse phenotyping in the patient and the family. We compared the prevalence of such cases in SRNS patients with that of SSNS controls, and analyzed their specific phenotypic features, response to therapies, and disease outcome.

Genetic analysis with whole-exome sequencing

We evaluated 110 consecutive patients with a diagnosis of "idiopathic nephrotic syndrome" before the age of 30 years. These included 63 patients with SRNS and 47 patients with SSNS. All cases were apparently sporadic and isolated at onset. All the patients underwent genetic testing by WES followed by *in silico* analysis. Identified variants were prioritized based on the algorithm shown in Figure 7, in agreement with the American College of Medical

Genetics and Genomics (ACMG) guidelines (Richards S, 2015). We confirmed all variants by Sanger sequencing and evaluated segregation in the family of all patients (Figure 7, Table 4). In addition, each variant frequency was further validated in a group of 300 Italian caucasian controls. Indeed, the absence also in this control group of all the identified variants supported their pathogenicity in our patients that were over 90% caucasian Italians (Table 4).

To detect phenocopies and exclude selection bias in the choice of the genes we analyzed all 284 genes reported until now as even rare causes of CKD, even if not usually associated with NS (Figure 8A, Table 3). WES identified 65 potentially pathogenic variants in 38/63 SRNS patients (Figure 8B). In agreement with previous results, 33.3% (21/63) of SRNS patients showed potentially pathogenic variants in known podocyte genes (Figure 8B). However, 11.1% (7/63) had potentially pathogenic variants in genes of the glomerular basement membrane (GBM), such as collagen IV or laminin, and 15.9% (10/63) in genes related to CAKUT, ciliopathies, tubulopathies or other rare nephropathies (Figure 8B). The remaining SRNS patients did not carry any potentially pathogenic variant within the 284 nephropathic genes (Figure 8B). Moreover, 15.9% (10/63) of SRNS patients carried variants of unknown significance (undefined) and were considered as negative but potentially including unrecognized genetic patients and thus treated as a separate group in the further statistical analysis (Figure 8B). Indeed, only 23.8% (15/63) of SRNS patients were completely negative (Figure 8B). Likewise, SSNS patients did not carry either potentially pathogenic variants or variants of unknown significance

(Figure 8C). Interestingly, over 73% of patients had been diagnosed with primary SRNS and treated accordingly in at least two independent nephrology units and 15.9% in at least three. These results suggest that almost 1/3 of SRNS patients does not carry potentially pathogenic variants in podocyte genes, but rather in genes related to CAKUT, ciliopathies, GBMopathies (GBMP) or other genetic nephropathies.

Reverse phenotyping and personalized genotype-phenotype correlations

To confirm those genetic diagnoses that were unexpected or uncertain because of apparent absence of familiarity, we moved back to clinical observation of patients. We then looked for signs referable to the genetic disease discovered by WES ("reverse phenotyping") and performed a deep phenotyping of each patient and of his/her relatives (Table 5). With this strategy, the following unexpected or uncertain diagnoses were addressed:

Case 12. The patient presented with SRNS and showed FSGS at biopsy. Upon identification of a potentially pathogenic variant in *ANLN*, an AD-transmitted gene inherited from the apparently healthy father, reverse phenotyping identified the presence of a mild proteinuria (300-500 mg/day) in the father.

Case 17. The patient developed a severe NS resistant to steroids and calcineurin inhibitors. Although WES identified a potentially pathogenic variant in *LMX1B*, no signs of Nail Patella syndrome were present apart from NS, consistently with previous reports (Boyer O, 2013). Reverse phenotyping by X-rays showed a bilateral lack of the nucleus of ossification of the radium that was the only bone abnormality related to the syndrome. In addition, the patient had normal nails,

but the lunula was absent from the thumbs. The variant was inherited from the apparently healthy father that, upon reverse phenotyping, showed microscopic hematuria and mild proteinuria (300-500 mg/day) (Giglio S, 2015).

Case 22-24. These three patients showed SRNS but no signs of Alport syndrome at biopsy, being diagnosed with FSGS (case 22 and 23), or minimal change disease (MCD) (case 24). In addition, all patients were checked for sensorineural hearing loss (SNHL) at diagnosis of NS with normal findings. Case 22 developed SNHL later and was diagnosed 8 years after onset when rechecked upon WES assessment, while case 23 and 24 still show no SNHL ten years after onset. Case 24 was treated not only with steroids but also with cyclosporine, tacrolimus and MMF, with no response. WES identified in case 22 and 23 potentially pathogenic variants in the AR-transmitted *COL4A4* and case 24 in *COL4A3.* The phenotype was consistent with previous case reports describing NS and FSGS without other sign of Alport syndrome in patients with collagen mutations (Malone AF, 2014). Variants were inherited by each of the apparently healthy parents that upon reverse phenotyping were found to have persistent microscopic hematuria without or with a mild proteinuria (300-500 mg/day). Parents of case 22 were not consanguineous but came from an inbred population of a small African village. Case 22 also carried *APOL1* G1/G2 risk alleles (Genovese G, 2010) explaining her rapid progression toward ESRD (about 7 years from the onset).

Case 25-27. The three patients showed SRNS and no signs of Alport syndrome at biopsy, being diagnosed with FSGS (case 25 and 27) or MCD (case 26). WES

performed several years after onset identified potentially pathogenic variants in the X-linked dominant *COL4A5* inherited by the respective mothers in all three cases. Reverse phenotyping in family members revealed that in case 25, the mother did not show renal involvement, but had SNHL and in case 26 had a microhematuria. In case 27, the mother had developed a NS during pregnancy that regressed but did not completely resolve after delivery.

Case 28. The patient showed SRNS and was referred for a second opinion on cyclosporine treatment. The biopsy showed FSGS. WES revealed compound heterozygous potentially pathogenic missense variants in the AR-transmitted *LAMB2*. *LAMB2* deletion cause Pierson syndrome including SRNS, but missense mutations are associated with isolated kidney involvement as shown in a previous report (Matejas V, 2010). The patient also showed compound heterozygous variants in the AR-transmitted *COL4A4 (*c. [2899A>G];[3914C>T] p.[Ile967Val];[Pro1305Leu]). All the variants were consistent with segregation in the unaffected parents. Coinheritance of *LAMB2* and *COL4A4* likely resulted in aggravation of the phenotype consistent with a recent report (Funk SD, 2018). The patient had an older sister that had been diagnosed with and treated for mesangioproliferative glomerulonephritis in another center. Genetic analysis of the sister confirmed that she also carried the same *LAMB2* variants.

Case 29. The patient was born from an oocyte donation. She showed signs of NS discovered during a check-up for a mild learning delay at 2 years of age. During the check-up she was checked for SNHL with negative results. Biopsy showed FSGS. She underwent treatment first with steroids and later with

cyclosporine for almost four years with no response. Four years after onset she developed SNHL and she underwent WES. No mutations in podocyte and Alport genes were observed. However, WES showed three potentially pathogenic variants, compound heterozygous in the AR-transmitted *CEP290/NPHP6* and a third heterozygous variant in *NPHP4* (Helou J, 2007) (Chaki M, 2011) (M'hamdi O, 2014). Only one *CEP290/NPHP6* potentially pathogenic variant was inherited by the healthy father. These genes cause nephronophtisis-related ciliopathies and can cause SNHL and mental retardation, and cases of digenic inheritance involving these two genes have already been reported (Helou J, 2007). Reverse phenotyping showed cortical dishomogeneity and thinning with hyperechoic areas not evidenced by the previous ultrasound performed at 2 years of age. Renal scintigraphy showed reduced global renal function with asymmetry.

Case 30. The patient showed a severe isolated NS, with diagnosis of FSGS at biopsy. After steroids, she was also treated with cyclosporine, MMF, tacrolimus, rituximab and plasmapheresis with no response. WES evidenced the "D313Y" variant in *GLA* (Koulousios K, 2017), a pseudodeficiency allele causative for organ-specific Fabry disease, often with distinct organ involvement in different members of the same family (Koulousios K, 2017). Reverse phenotyping showed lysosomal accumulation and vacuolization in podocytes with extremely rare and small concentric lamellary bodies in the kidney biopsy (Koulousios K, 2017). The variant was inherited from the apparently healthy father that showed decreased α-Gal A activity and only

upon reverse phenotyping was diagnosed with high frequency SNHL, observed in 60% of subjects with Fabry disease, particularly hemizygous males (Köping M, 2017).

Case 31. The patient showed NS and intermittent microhematuria, and a history of cluster headache. Renal biopsy showed FSGS with tubular atrophy. Genetic testing revealed rare homozygous potentially pathogenic variants in the AR-transmitted *FAT1*, in agreement with a recent report (Gee HY S. C., 2017). The variants were inherited by non-consanguineous healthy parents coming from an inbred village of southern Italy.

Case 32. The patient showed mild NS. Renal ultrasound scanning at onset was unremarkable. She showed compound heterozygous potentially pathogenic variants in the AR-transmitted *FAT4*, known as a CAKUT gene causing kidney hypoplasia. Reverse phenoyping with renal scintigraphy revealed a left renal hypodysplasia (van der Ven AT, 2017).

Case 33. The patient showed NS and a missense *de novo* potentially pathogenic variant in AD-transmitted *PAX2* (Barua M, 2014). Reverse phenotyping confirmed that the NS was isolated. Consistently, deletions of *PAX2* are associated with the renal-coloboma syndrome, while missense mutations, including the one observed in the patient, are associated with isolated FSGS and SRNS (Okumura T, 2015).

Case 34. The patient was found to have proteinuria in the nephrotic range and a mild CKD during a checkup for lipothymia episodes. The biopsy showed FSGS and the patient was treated with steroids, calcineurin inhibitors, MMF, and

rituximab with no response. She was referred to our unit for genetic testing and a second opinion when she had already reached CKD stage IV. WES showed compound heterozygous potentially pathogenic variants in one of the genes of Fraser syndrome, *FRAS1,* inherited by the healthy parents. As reported, missense mutations in *FRAS1* cause isolated CAKUT while deletions are associated with the full Fraser syndrome phenotype (Kohl S, 2014). Moreover, she showed another potentially pathogenic variant in *FREM2*. The proteins encoded by *FREM2* and *FRAS1* form the same protein complex and can both cause Fraser syndrome (Kohl S, 2014). For reverse phenotyping after the genetic diagnosis we reconsidered the left ureteric junction duplication that was observed at ultrasound and initially overlooked as an incidental finding. Patient history revealed untreated relapsing urinary tract infections as well as untreated high fevers of unknown origin. Re-evaluation of the kidney biopsy evidenced some signs of adaptive FSGS such as perihilar lesions, periglomerular fibrosis, and interstitial infiltration of neutrophils, suggesting secondary FSGS related to the genetic CAKUT and chronic pyelonephritis (Sethi S, 2014). The patient reached ESRD five years after diagnosis turning progressively more nephrotic, as reported for some CAKUT cases (Wong CS P. C., 2009). She carried also a variant of unknown clinical significance in *ACTN4* inherited by the healthy father that may have aggravated proteinuria.

Cases 35-37. All the patients were severely nephrotic at onset and showed FSGS (case 35 and 37) or MCD (case 36) at biopsy. A mild tubular proteinuria was observed together with massive albuminuria, but did not generate suspicion

because mild tubular proteinuria is frequent in patients with FSGS because of proximal tubular injury (Vallés P, 2000). Patients 36 and 37 were also treated with calcineurin inhibitors with no response. WES showed a potentially pathogenic variant in X-linked recessive-transmitted *CLCN5* in all patients. No other signs of tubular dysfunction (glycosuria, hypercalciuria, alkaline urinary Ph) appeared over time in two patients, while one later developed hypercalciuria. This is in agreement with a recent report showing that 50% of patients affected by Dent disease present with nephrotic proteinuria and FSGS, in absence of hypercalciuria (van Berkel Y, 2017) (Wang X, 2016).

Case 38. The patient showed isolated NS with a severe albuminuria. Polyuria, hematuria, low-molecular weight proteinuria or other signs of tubular dysfunction were present. Renal ultrasound scanning and renal scintigraphy were both normal at onset. Kidney biopsy showed MCD. WES showed two potentially pathogenic variants in *CTNS* inherited by each of the parents who are not consanguineous. Upon reverse phenotyping, slit-lamp examination revealed symmetric corneal crystals. The half-cysteine levels in peripheral blood polymorphonuclear cells were elevated. Finally, re-evaluation of the biopsy showed several multinucleated podocytes. In agreement with previous studies, nephropathic cystinosis may clinically appear as a podocytopathy, with nephrotic-range proteinuria or frank nephrotic syndrome (Chandra M, 2010) (Bonsib SM, 1999) (Dhooria GS, 2014), sometimes without any sign of tubular dysfunction or extrarenal signs (Servais A, 2008). In conclusion, WES

and reverse phenotyping revealed an unexpectedly high rate of phenocopies in patients with apparently sporadic and non-syndromic SRNS.

Outcome prediction

We next questioned how patients responded to treatment in relation to the genetic diagnosis. All SSNS patients underwent complete remission in response to steroids or to IS used as steroid-sparing agents. Negative SRNS patients, i.e. patients for which no genetic cause could be identified, responded in 86.6% with either complete or partial remission (53.3% and 33.3% respectively). Strikingly, among all SRNS patients, complete remission occurred exclusively in WES-negative patients (Table 5). By contrast, all treated phenocopies were resistant to IS treatment (Table 5), consistently with the genetic diagnosis.

Next, we correlated WES results with long-term renal outcome. SSNS patients showed 100% renal survival after 10 years of follow-up. Patients with podocytopathies had a 10-years kidney survival rate of 39% (Table 5, Figure 9). By contrast, negative patients and phenocopies showed an outcome significantly different from podocytopathies, with a survival rate of 75 and 78% at 10 years, respectively (Figure 9). Of note, undefined patients also showed a poor outcome, with a rate of renal survival of only 20% at 10 years.

Finally, among 20 SRNS patients who underwent renal transplant, none of the patients with a clear genetic diagnosis developed recurrent post-transplant NS (0/12 patients, 0/15 grafts), while patients with undefined SRNS had developed recurrent NS in 57% (4/7 patients, 5/8 grafts). Together,

phenocopies represent a significant proportion of patients with SRNS that will not benefit from immunosuppressive treatment and who are at a higher risk to develop post-transplant recurrence of NS.

Discussion

We had hypothesized that among patients with the clinical syndrome of SRNS, phenocopies of genetic kidney disorders other than the well-known podocytopathies, may be more frequent than previously thought. By using WES and reverse phenotyping we found that: (i) WES-based genotyping together with reverse phenotyping of the patient and family members for an extended panel of nephropathic genes doubles the current diagnostic rate in SRNS because phenocopies altogether are as frequent as genetic podocytopathies in SRNS (30%); (ii) Patients with phenocopies are usually multi-drug resistant but have a better prognosis than podocytopathies.

To evaluate the prevalence of phenocopies in young patients with SRNS we used an unbiased comprehensive approach, based on WES and filtering for 284 genes responsible for genetic nephropathies and CKD, followed by a rigorous bioinformatic strategy. In particular, we involved not only databases but also a population of local controls, as well as segregation of the variants in the family, for the selection of potentially pathogenic variants. We then performed reverse phenotyping in search of minor or overlooked signs of the suspected underlying genetic disorders in the patient or other family carriers, a step that permitted to confirm genetic diagnosis in many cases. As a further level of

validation, we also applied an identical diagnostic strategy to a cohort of 47 patients with SSNS that instead all appeared as negative, consistent with recent reports of genetic inheritance related to alterations of the immune system and not of kidney genes (Dorval G, 2018) (Gbadegesin RA, 2015).

So far, genetic screening strategies based on podocyte gene panels sequencing identified a genetic etiology in about 30% of children with SRNS (Büscher AK B. B., 2014) (Giglio S, 2015) (Sadowski CE & SRNS Study Group, 2015) (Wang F Z. Y., 2017) (Bierzynska A, 2017) (Trautmann A B. M., 2015), a percentage that in our study remained unchanged even with the applied strategy. However, the majority of the remaining patients displayed potentially pathogenic variants in genes causing other nephropathies, including GBMP, CAKUT, ciliopathies and tubulopathies, explaining the heterogeneous prognosis. Interestingly, virtually all these patients showed a histopathological diagnosis of FSGS, irrespective of the genotype. This is consistent with FSGS lesions, as well as NS, having been reported in cases of Alport syndrome, CAKUT, ciliopathies and Dent's Disease (Barua M, 2014) (Gast C, 2016) (Pierides A, 2009) (Fogo AB, 2010) (Saida K, 2010) (Bullich G, 2017) (Toka HR1, 2010) (Fervenza FC, 2013) and with previous observations showing that many patients with CAKUT become nephrotic during the long-term course of the disease (Sethi S, 2014). In none of these patients, the true diagnosis had been suspected based on the clinical presentation, which was that of an isolated, sporadic SRNS and on biopsy results. Indeed, biopsy results of FSGS or in some cases of MCD did not trigger further diagnostic work-up. These observations explain why SRNS was invariably diagnosed and steroid treatment was started, often followed by

multiple IS treatments that could have been avoided if the correct diagnosis would have been available. In contrast to WES-negative patients, phenocopies were multi-drug resistant, but had in general a better outcome than podocytopathies. No complete remissions were observed, while partial responses to treatments were observed only in a minority of them and usually only to inhibitors of the renin-angiotensin-aldosterone system (RASi). Finally undefined patients, who represented 15.9% (10/63) of SRNS in our cohort, usually also recognized as negative in clinical practice, had a significantly different outcome from negative patients, likely because they anyway included not only unrecognized genetic cases but also patients with particularly severe disorders related to some permeability factor (D'Agati VD, 2011) (Gallon L, 2012). Consistently, these patients not only frequently progressed toward ESRD, but also showed a high risk of NS recurrence after kidney transplant (Gallon L, 2012) (Francis A, 2016). By contrast, genetic patients showed a negligible risk of post-transplantation recurrence of disease, in agreement with previous results (Bierzynska A, 2017) (Trautmann A B. M., 2015).

Taken together, the results of this study imply that in young patients with SRNS the existence of a phenocopy caused by an underlying genetic kidney disorder other than podocytopathies should always be carefully considered during the diagnostic work-up to: 1. Define the underlying cause of disease; 2. Guide the clinical management; 3. Better predict the prognosis; 4. Predict the recurrence of NS after kidney transplant; 5. Enable genetic counseling for double as many affected families than before.

These results advocate WES-based genetic testing for a wider spectrum of nephropathic genes together with reverse phenotyping to identify the numerous patients with unexpected diagnosis in patients with idiopathic SRNS.

Figures and Tables

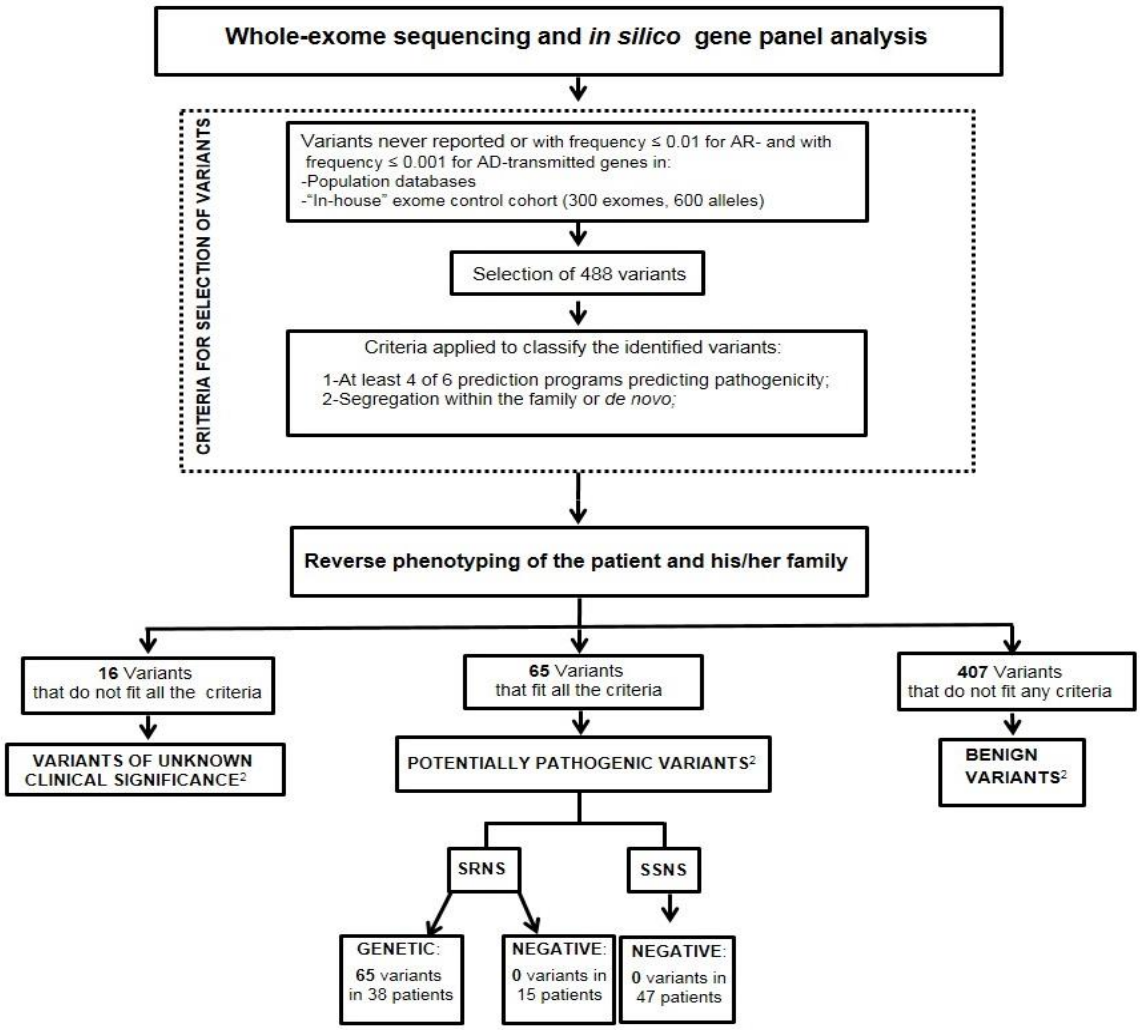

Figure 7. Flow chart for variants prioritization.

Flow chart showing the diagnostic yield resulting from WES and semiautomatic bioinformatic analysis of 284 nephropathic genes in 110 patients with idiopathic NS. Following the prioritization algorithm that includes reverse phenotyping of the patients and the family members, the identified variants were classified as potentially pathogenic variants, variants of unknown clinical

significance, or benign variants, in agreement with the interpretation guidelines of the ACMG (Richards S, 2015).

AR, autosomal recessive; AD, autosomal dominant; SRNS, steroid-resistant nephrotic syndrome; SSNS, steroid-sensitive nephrotic syndrome.

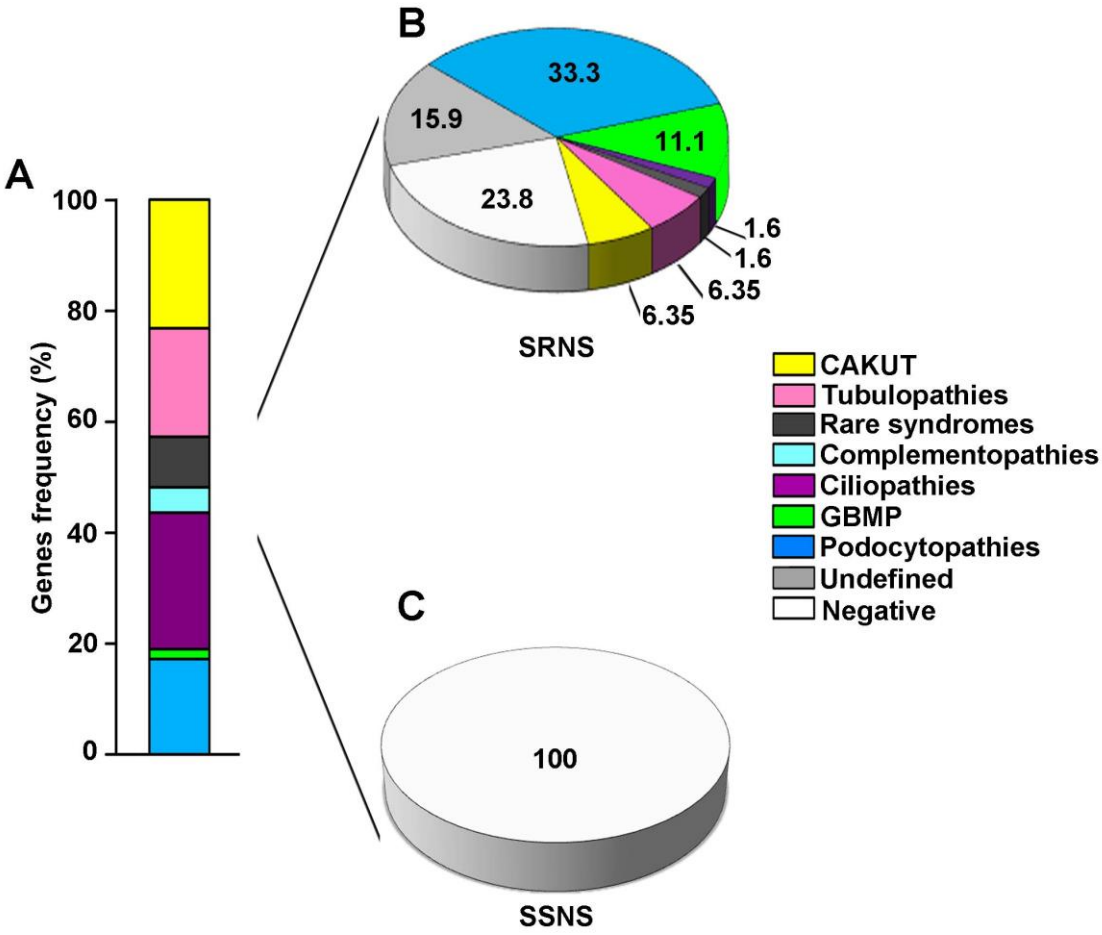

Figure 8. WES unravels heterogeneous genotypes in SRNS but not in SSNS patients.

(A) Panel of 284 nephropathic genes analyzed by WES in all the patients as grouped in the following families: 1. Podocytopathies (blue); 2. Diseases related to the glomerular basement membrane (GBMopathies, GBMP, green); 3. Ciliopathies (purple); 4. Complementopathies (light blue); 5. Rare syndromes with kidney involvement (gray); 6. Tubulopathies (pink); and 7. Congenital abnormalities of the kidney and urinary tract (CAKUT, yellow).

(B) Frequency of patients carrying potentially pathogenic variants in the different families of genes identified. (C) Results of the same analysis in SSNS patients yielding identification of no potentially pathogenic variants. Patients that did not carry any potentially pathogenic variants are in white in all the panels.

SRNS, steroid-resistant nephrotic syndrome; SSNS, steroid-sensitive nephrotic syndrome; CAKUT, congenital abnormalities of the kidney and urinary tract; GBMP, diseases related to the glomerular basement membrane.

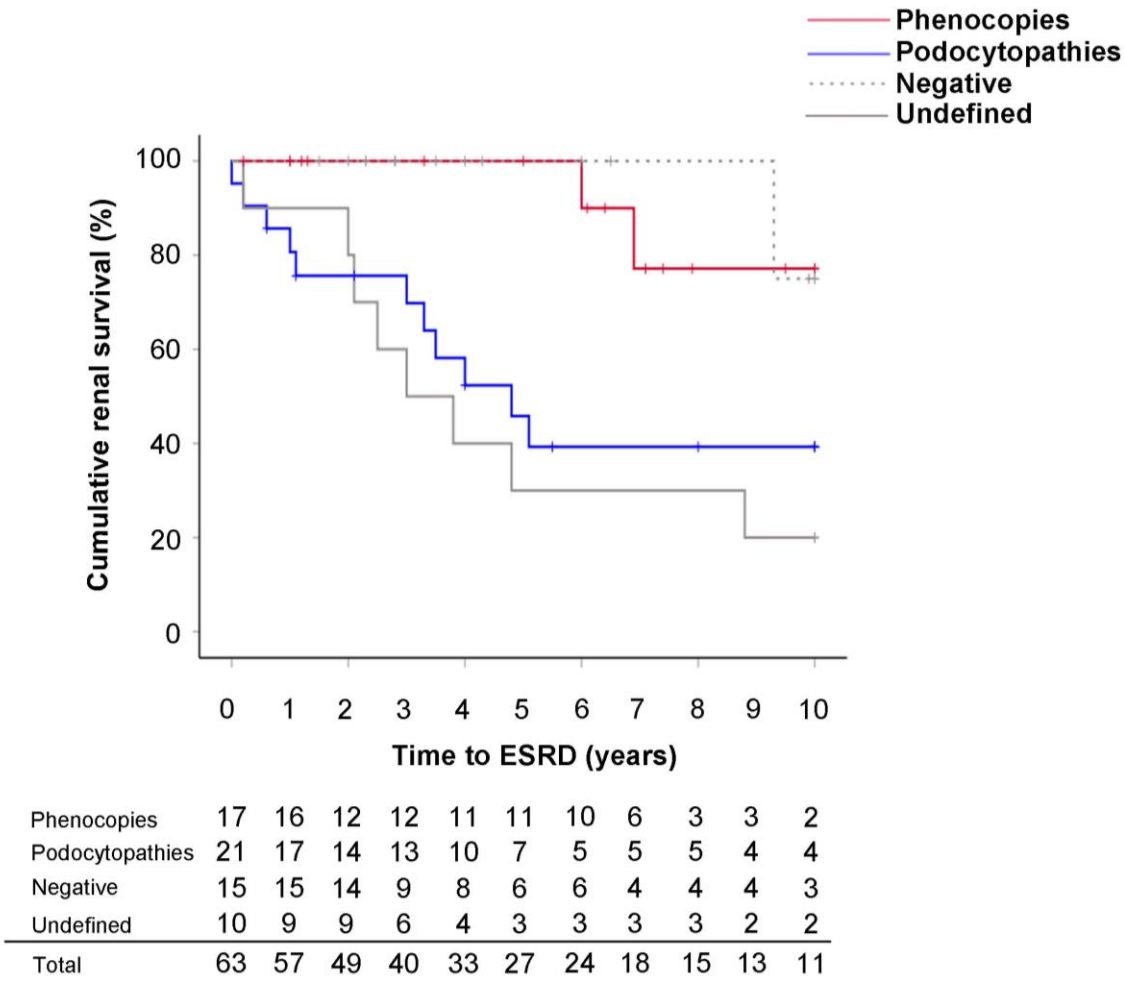

Figure 9. Renal survival of patients according to the genetic diagnosis.

Kaplan-Meier renal survival analysis comparing the rates of ESRD development over a period of 10 years from the diagnosis in patients with SRNS stratified according to WES results. Length of follow-up was similar between groups (ANOVA p=0.75).

ESRD, end-stage renal disease.

Renal survival rates were compared between groups by log rank test:

Podocytopathies *vs* Phenocopies, log rank 7.03 $p<0.01$

Podocytopathies *vs* Negative, log rank 6.43 $p<0.05$

Undefined *vs* Phenocopies, log rank 9.08 $p<0.01$

Undefined *vs* Negative, log rank 9.45 $p<0.01$

Table 3. Panel of 284 genes analyzed with WES.

Gene	Locus
ACE	chr17:61554422-61575741
AGT	chr1:230838272-230850336
AGTR1	chr3:148415658-148460790
AGTR2	chrX:115301958-115306225
AIFM3	chr22: 21319396-21335649
ALG1	chr16: 5083703-5137380
BMP4	chr14:54416455-54421270
BMP7	chr20:55743809-55841707
CDC5L	chr6:44355251-44418161
CHD1L	chr1:146714291-146767447
CHD7	chr8: 61591337-61779465
CHRM3	chr1: 239549865-240078750
CRKL	chr22: 21271714-21308037
DCHS1	chr11: 6642556-6677085
DCHS2	chr4: 155153399-155412930
DSTYK	chr1:205111631-205180727
E2F3	chr6:20402398-20493941
EXT1	chr8: 118806729-119124092
EYA1	chr8:72109668-72268979
FAT1	chr4: 187508937-187647876
FAT3	chr11: 92085262-92629618
FAT4	chr4: 126237554-126414087
FGF20	chr8:16850334-16859674
FGFR1	chr8:38268656-38325363
FGFR2	chr10:123237844-123357972
FOXI1	chr5: 169532901-169536727
FOXP1	chr3:71003844-71633140
FRAS1	chr4:78978724-79465423
FREM1	chr9: 14734664-14910993
FREM2	chr13:39261173-39461267
GATA3	chr10:8096667-8117164
GDNF	chr5: 37812779-37839788
GLI3	chr7:42000548-42276618
GRIP1	chr12:66741211-67072925
HNF1B	chr17:36046434-36105096
HPSE2	chr10:100216834-100995632
ITGA8	chr10: 15555948-15762124
ITGB1	chr10: 33189247-33294720
LRIG2	chr1:113615831-113667342
LRP4	chr11:46878419-46940193
MUC1	chr1:155158300-155162706
NRIP1	chr21: 16333556-16437321
OSR1	chr2:19551246-19558372
PAX2	chr10:102505468-102589697
PBX1	chr1: 164524821-164868533
REN	chr1:204123944-204135465
RET	chr10:43572517-43625797
ROBO2	chr3:77089294-77699114
SALL1	chr16:51169886-51185183
SALL4	chr20:50400583-50419048
SHH	chr7:155595558-155604967
SIX1	chr14:61111417-61116155
SIX2	chr2:45232324-45236542
SIX5	chr19:46268043-46272497
SLIT2	chr4:20255235-20620788
SNAP29	chr22:21213271-21245506
SOX17	chr8:55370495-55373456
SOX9	chr17:70117161-70122560

SPRY2	chr13: 80910111-80915086
TBX18	chr6: 85397069-85474237
TBX3	chr12:115108059-115121969
TNXB	chr6: 32008931-32083111
TRAP1	chr16:3708038-3767598
UMOD	chr16:20344373-20364037
UPK3A	chr22:45680868-45691755
USF2	chr19:35759896-35770718
ACTN4	chr19:39138327-39221170
ADCK4	chr19:41197434-41222790
ANLN	chr7:36429432-36493400
APOL1	chr22:36649117-36663577
ARHGAP24	chr4:86396284-86923823
ARHGDIA	chr17:79825597-79829282
CD151	chr11:832952-838834
CD2AP	chr6:47445525-47594994
CDK20	chr9: 90581356-90589668
COQ2	chr4:84184979-84205964
COQ6	chr14:74416955-74429813
CRB2	chr9:126118448-126141032
DGKE	chr17:54911460-54946036
DLC1	chr8: 12940870-13373167
EMP2	chr16:10622279-10674539
GPC3	chrX:132669776-133119673
GPC5	chr13:92050935-93519487
INF2	chr14:105155943-105185947
ITGA3	chr17:48133340-48167848
ITGB4	chr17:73717516-73753899
ITSN1	chr21: 35014706-35272165

ITSN2	chr2: 24425733-24583583
KANK1	chr9: 470291-746105
KANK2	chr19: 11274943-11308467
KANK4	chr1: 62702651-62785085
LMX1B	chr9:129376748-129463311
MAGI2	chr7: 77646393-79082890
MYH9	chr22:36677324-36784063
MYO1E	chr15:59428564-59665071
NPHS1	chr19:36316274-36342739
NPHS2	chr1:179519677-179545084
NUP107	chr12:69080514-69136785
NUP205	chr7:135242667-135333505
NUP93	chr16: 56764017-56878797
NXF5	chrX:101087085-101112549
PDSS2	chr6:107473761-107780779
PLCE1	chr10:95753746-96088146
PODXL	chr7:131185023-131241376
PTPRO	chr12:15475487-15750335
SCARB2	chr4:77079894-77135035
SGPL1	chr10: 72575717-72640930
SMARCAL1	chr2:217277473-217347772
SYNPO	chr5:150020220-150038792
TENC1	chr12:53440753-53458156
TRPC6	chr11:101322296-101454659
WT1	chr11:32409325-32457087
XPO5	chr6: 43490072-43543812
ADAMTS13	chr9:136287120-136324508
C3	chr19:6677846-6720662
CD46	chr1:207925383-207968861

CFB	chr6: 31895475-31919861
CFH	chr1:196621008-196716634
CFHR1	chr1:196743930-196763203
CFHR2	chr1: 196788898-196928356
CFHR3	chr1:196743930-196763203
CFHR4	chr1: 196819371-196888102
CFHR5	chr1: 196946667-196978804
CFI	chr4:110661848-110723335
PLG	chr6: 161123270-161174347
THBD	chr20:23026270-23030301
AGXT	chr2: 241807896-241819919
CREBBP	chr16:3775056-3930121
DHCR7	chr11:71145457-71159477
DIS3L2	chr2:232826293-233201908
DLL4	chr15:41221531-41231258
FGF10	chr5: 44303646-44389808
GLA	chrX: 100652791-100662913
GRHPR	chr9: 37422663-37436987
HOGA1	chr10: 99344080-99372559
JAG1	chr20:10618332-10654694
KAL1	chr6:20534688-21232634
KCNJ10	chr1: 160007257-160040038
NARS2	chr11: 78147007-78285919
NEU1	chr6: 31825436-31830683
NOTCH2	chr1:120454176-120612317
OCRL	chrX: 128673826-128726538
PEX1	chr7: 92116334-92157845
PMM2	chr16:8891670-8943194
ROR2	chr9: 94325373-94712444
TFAP2A	chr6: 10393419-10419892

VIPAS39	chr14: 77893018-77924295
VPS33B	chr15: 91541646-91565833
WDR73	chr15:85186012-85197521
WNT4	chr1:22443798-22469519
WNT5A	chr3: 55499743-55523973
ZMPSTE24	chr1:40723779-40759856
AHI1	chr6: 135604670-135818914
ALDOB	chr9:104182860-104198105
ALMS1	chr2: 73612886-73837920
ANKS6	chr9: 101493611-101559247
ARL13B	chr3: 93698983-93774512
ARL6	chr3: 97483365-97519953
ATXN10	chr22: 46067678-46241187
B9D1	chr17: 19240867-19281495
B9D2	chr19: 41860326-41870078
BBS1	chr11: 66278077-66301098
BBS2	chr16: 56500748-56554195
BBS4	chr15: 72978527-73030817
BBS5	chr2: 170335688-170382432
BBS7	chr4: 122745595-122791652
C14ORF17 9/ IFT43	chr14: 76368479-76550928
C4ORF24/ BBS12	chr4: 123653857-123666097
C5ORF42	chr5: 37106330-37249530
CC2D2A	chr4: 15471489-15603180
CEP164	chr11: 117185273-117283984
CEP290	chr12: 88442793-88535993
CEP41	chr7: 130033612-130082274
CEP83/	chr12: 94700225-94853764

CCDC41	
DYNC2H1	chr11: 102980160-103350591
EVC	chr4: 5712924-5830772
EVC2	chr4: 5544499-5711275
FAN1	chr15: 31196055-31235311
GLIS2	chr16: 4364762-4389598
IFT122	chr3: 129158968-129239198
IFT140	chr16: 1560428-1662111
IFT172	chr2: 27667238-27712656
IFT80	chr3: 159974774-160117668
INPP5E	chr9: 139323071-139334274
INVS	chr9: 102861538-103063282
IQCB1	chr3: 121488610-121553926
KIF14	chr1: 200520628-200589862
KIF7	chr15: 90152020-90198682
LZTFL1	chr3: 45864808-45957534
MKKS	chr20: 10381657-10414870
MKS1	chr17: 56282803-56296966
NEK1	chr4: 170314426-170533780
NEK8	chr17: 27052915-27070473
NPHP1	chr2: 110879888-110962643
NPHP3	chr3: 132276986-132441303
NPHP4	chr1: 5922871-6052533
OFD1	chrX:13752832-13787480
PDE6D	chr2: 232597135-232650982
PKHD1	chr6: 51480098-51952423
POC1A	chr3: 52109269-52188706
PTHB1/BBS9	chr7: 33168856-33645680
RPGRIP1L	chr16: 53631595-53737850
SCLT1	chr4: 129786076-130014764
SDCCAG8	chr1: 243419320-243663394
TBC1D32	chr6: 121400640-121655891
TCTN1	chr12: 111051832-111087235
TCTN2	chr12: 124155660-124192948
TMEM138	chr11: 61129473-61136981
TMEM216	chr11: 61159159-61166335
TMEM231	chr16: 75572015-75590184
TMEM237	chr2: 202484907-202508293
TMEM67	chr8: 94767072-94831462
TRIM32	chr9: 119449581-119463579
TTC21B	chr2:166729872-166810348
TTC8	chr14: 89290497-89344335
WDPCP	chr2: 63348518-64054977
WDR19	chr4: 39184024-39287430
WDR34	chr9: 131395940-131419066
WDR35	chr2: 20110021-20189892
WDR60	chr7: 158649269-158749438
XPNPEP3	chr22: 41253081-41363838
ZNF423	chr16: 49521435-49891830
AQP2	chr12: 50344524-50352664
AQP6	chr12: 50360977-50370922
ATP6V0A4	chr7:138391040-138484305
ATP6V1B1	chr2:71163012-71192536
ATP6V1C2	chr2:10861775-10925236
AVP	chr20: 3063202-3065370
AVPR2	chrX: 153167985-153172620
BCS1L	chr2: 219523487-219528166
BSND	chr1: 55464606-55476556

CA2	chr8: 86376081-86393722
CASR	chr3: 121902530-122005342
CLCN5	chrX: 49687225-49863892
CLCNKA	chr1: 16345370-16360545
CLCNKB	chr1: 16370272-16383803
CLDN16	chr3: 190040330-190129932
CLDN19	chr1: 43198764-43205925
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CTNS	chr17: 3,539,762-3,564,836
CUBN	chr10:16865965-17171816
CUL3	chr2: 225334867-225450110
EGF	chr4: 110834040-110933422
EHHADH	chr3: 184908412-184999778
FXYD6-FXYD2	chr11: 117690878-117747382
G6PC	chr17: 41052814-41065386
HNF4A	chr20: 42984340-43061485
KCNJ1	chr11:128706210128737268
KLHL3	chr5: 136953189-137071779
MRPS22	chr3: 138724648-139076065
NR3C2	chr4: 148999913-149365850
OSGEP	chr14: 20914570-20923264
RRM2B	chr8: 103216730-103251346
SCNN1A	chr12: 6456009-6486896
SCNN1B	chr16: 23289552-23392620
SCNN1G	chr16: 23194036-23228204
SLC12A1	chr15: 48483861-48596275
SLC12A3	chr16: 56899119-56949762

SLC2A2	chr3: 170714137-170744539
SLC34A1	chr5: 176806236-176825849
SLC36A2	chr5: 150694539-150727151
SLC3A1	chr2: 44502599-44548633
SLC4A1	chr17: 42325753-42345509
SLC4A4	chr4: 72053003-72437804
SLC4A5	chr2: 74443369-74570541
SLC4A8	chr12: 51785101-51902980
SLC4A9	chr5: 139739787-139754728
SLC5A2	chr16: 31494323-31502181
SLC6A18	chr5: 1225470-1246304
SLC6A19	chr5: 1201710-1225232
SLC6A20	chr3: 45796942-45838027
SLC7A7	chr14:23242431-23299029
SLC7A9	chr19: 33321415-33360672
TP53RK	chr20: 45313004-45318418
TRPM6	chr9:77337411-77503010
WNK1	chr12: 861759-1020618
WNK4	chr17: 40932696-40948954
COL4A3	chr2:228029281-228179508
COL4A4	chr2:227867427-228029275
COL4A5	chrX:107683074-107940775
LAMB2	chr3:49158548-49170599
FN1	chr2: 216225163-216300895

Total number of genes: 284

CAKUT genes (yellow): 66

Podocytopathies genes (blue): 47

Complementopathies genes (light blue): 13

Rare syndrome genes (grey): 26

Ciliopathies genes (purple): 70

Tubulopathies genes (pink): 57

Glomerular basement membrane diseases genes (green):

Table 4. Genetic profile of patients included in the study.

Patient	Gender	Ethnicity	POTENTIALLY PATHOGENIC VARIANTS							
			Gene	**MOI**	**Nucleotide change**	**Amino acid change**	**Inheritance**	**gnomAD allele frequency**	**In-house control cohort** (300 WES)	**Reference**
Case 1	M	Caucasian	*NPHS2*	AR	c.413G>A c.467_468dupT	p.Arg138Gln p.Leu156PhefsTer10	mat pat	0.00115 0.00083	NP NP	-Laurin et al. Nephrol Dial Trans. 2014. -Wang et al. Pediatr Nephrol 2017
Case 2	M	Caucasian	*NPHS2*	AR	c.104dupG c.1143delC	p.Arg36ProfsTer33 p.Met382CysfsTer?	mat pat	NP NP	NP NP	-Boute et al. Nat Genet, 2000 -Giglio et al. J Am Soc Nephrol 2015
Case 3	F	Caucasian	*NPHS2*	AR	c.686G>A c.911C>T	p.Arg229Gln p.Ser304Phe	mat pat	0.03591 NP	NP NP	-Tory et al.Nat Genet 2014 -Giglio et al. J Am Soc Nephrol 2015
Case 4	M	Caucasian	*NPHS2*	AR	c.479A>G c.855_856delAA	p.Asp160Gly p.Arg286ThrfsTer16	mat pat	0.00000 0.00014	NP NP	-Boute et al. Nat Genet, 2000 -Boute et al. Nat Genet, 2000
Case 5	M	Caucasian	*NPHS2*	AR	c.365G>C c.416T>C	p.Trp122Ser p.Leu139Pro	mat pat	0.00000 NP	NP NP	-Bouchireb et al. Hum Mutat 2014 -ND
Case 6	M	Caucasian	*NPHS2*	AR	c.538G>A c.538G>A	p.Val180Met p.Val180Met	paternal uniparental isodisomy	0.00000	NP	-Boute et al. Nat Genet, 2000
Case 7	F	Caucasian	*NPHS2*	AR	c.419delG c.419delG	p.Gly140AspfsTer40 p.Gly140AspfsTer40	mat pat	NP	NP	-Boute et al. Nat Genet, 2000
Case 8	M	Caucasian	*NPHS2*	AR	c.686G>A c.883G>A	p.Arg229Gln p.Ala295Thr	mat pat	0.03591 NP	NP NP	-Tory et al.Nat Genet 2014 -Megremis et al. Gen Test Mol Bioma. 2009
Case 9	F	Caucasian	*NPHS2*	AR	c.946C>T c.686G>A	p.Pro316Ser p.Arg229Gln	pat mat	NP 0.03591	NP NP	-Sadowski et al. J Am Soc Nephrol 2015 -Tory et al. Nat Genet 2014.
Case 10	M	Caucasian	*NPHS2*	AR	c.467T>C c.855_856delAA	p.Leu156Ser p.Arg286ThrfsTer16	pat mat	NP 0.00014	NP NP	-ND -Boute et al. Nat Genet, 2000

Case 11	F	Caucasian	*NPHS1*	AR	c.2928G>T c.2299C>T	p.Arg976Ser p.Pro767Ser	pat mat	0.00007 0.00001	NP NP	-Lovric et al. Clin J Am Soc Nephrol 2014 -Cil et al. Pediatr Nephrol 2015
Case 12	M	Caucasian	*ANLN*	AD	c.745A>G	p.Asn249Asp	pat	NP	NP	-ND
Case 13	M	Caucasian	*PLCE1*	AR	c.325_326delAT c.4570_4572delATG	p.Leu110GlufsTer13 p.Met1524del	pat mat	0.00000 NP	NP NP	-ND -ND
Case 14	F	Caucasian	*PLCE1*	AR	c.4327G>A c.2038C>T	p.Gly1443Arg p.Gln680Ter	pat mat	NP NP	NP NP	-Giglio et al. J Am Soc Nephrol 2015
Case 15	F	Caucasian	*ACTN4*	AD	c.782T>A	p.Val261Glu	*de novo*	NP	NP	-Giglio et al. J Am Soc Nephrol 2015
Case 16	F	Caucasian	*ACTN4*	AD	c.464T>C	p.Ile155Thr	*de novo*	NP	NP	-Giglio et al. J Am Soc Nephrol 2015
Case 17	M	Tunisian	*LMX1b*	AD	c.764C>T	p.Ala255Val	pat	NP	NP	-Giglio et al. J Am Soc Nephrol 2015
Case 18	F	Caucasian	*WT1*	AD	c.1388C>T	p.Ser463Phe	*de novo*	NP	NP	-ND
Case 19	F	Caucasian	*WT1*	AD	c.1384C>T	p.Arg462Trp	*de novo*	0.00000	NP	-Lehnhardt et al. Clin J Am Soc Nephrol 2015
Case 20	M	Caucasian	*WT1*	AD	c.1300C>T	p.Arg434Cys	*de novo*	0.00000	NP	-Lehnhardt et al. Clin J Am Soc Nephrol 2015
Case 21	M	Caucasian	*KANK1*	AR	c.971A>G c.200G>A	p.Tyr324Cys p.Arg67Gln	*de novo* mat	0.00002 0.00628	NP NP	-ND
Case 22	F	Senegalese	*COL4A4* *APOL1*	AR AR	c.1445C>G c.1445C>G c.1024A>G c.1152T>G c.1164_1169delTTATAA	p.Pro482Arg p.Pro482Arg p.Ser342Gly p.Ile384Met p.Asn388_Tyr389del	mat pat mat mat pat	NP 0.22630 0.22600 0.14040	NP NP NP NP	-ND -Genovese et al. Science, 2010 -Genovese et al. Science, 2010 -Genovese et al. Science, 2010
Case 23	F	Caucasian	*COL4A4*	AR	c.3289+1G>A c.2590G>A	p.Gly864Arg	mat pat	NP NP	NP NP	-Xiong et al. Science.2015 -Storey et al. J Am Soc Nephrol 2013

Case 24	F	Caucasian	*COL4A3*	AR	c.4421T>C c.1831G>A	p.Leu1474Pro p.Gly611Arg	mat pat	0.00489 NP	NP NP	-Gast et al. Nephrol Dial Transplant 2015 -ND
Case 25	F	Caucasian	*COL4A5*	XLD	c.1912G>A	p.Gly638Ser	mat	0.00000	NP	-Plant et al. Hum Mutat 1999
Case 26	M	Caucasian	*COL4A5*	XLD	c.991G>A	p.Gly331Ser	mat	NP	NP	-ND
Case 27	F	Caucasian	*COL4A5*	XLD	c.3197G>A	p.Gly1066Asp	mat	NP	NP	-ND
Case 28	M	Caucasian	*LAMB2*	AR	c.4868G>A c.1931G>A	p.Gly1623Asp p.Arg644His	mat pat	NP 0.00023	NP NP	-ND -ND
Case 29	F	Caucasian	*CEP290* *NPHP4*	AR AR	c.6401T>C c.1991A>G c.1852G>A	p.Ile2134Thr p.Asp664Gly p.Glu618Lys	pat not pat not pat	0.00688 0.01308 0.01440	NP NP NP	-Eisenberger et al. PLoS One 2013 -ND -ND
Case 30	F	Caucasian	*GLA*	XLR/XLD	c.937G>T	p.Asp313Tyr	pat	0.004458	NP	-Koulousios et al.BMJ, 2017
Case 31	M	Caucasian	*FAT1*	AR	c.6823G>A c.6823G>A	p.Asp2275Asn p.Asp2275Asn	mat pat	0.00003	NP	-ND
Case 32	F	Caucasian	*FAT4*	AR	c.11265A>T c.11953T>A	p.Glu3755Asp p.Tyr3985Asn	pat mat	NP NP	NP NP	-ND -ND
Case 33	F	Caucasian	*PAX2*	AD	c.239C>T	p.Pro80Leu	de novo	NP	NP	-Barua et al. J Am Soc Nephrol 2014
Case 34	F	Caucasian	*FRAS1* *FREM2*	AR AR	c.160G>C c.6623T>C c.685C>T	p.Asp54His p.Leu2208Pro p.Arg229Cys	pat mat mat	0.00327 NP 0.00000	NP NP NP	-ND -ND -Kohl et al. J Am Soc Nephrol 2014

Case 35	M	Caucasian	*CLCN5*	XLR	c.749G>A	p.Gly250Asp	*mat*	NP	NP	-ND
Case 36	M	Caucasian	*CLCN5*	XLR	c.608C>G	p.Ser203Trp	NA	NP	NP	-ND
Case 37	M	Caucasian	*CLCN5*	XLR	c.781G>A	p.Gly261Arg	mat	NP	NP	-Tosetto et al. Clin Genet. 2009
Case 38	M	Caucasian	*CTNS*	AR	c.198_218del 17p13.2 (3505485_3560005)x1	p.Ile67_Pro73del *(CTNS included in deleted region, protein absent)*	mat pat	0.00003	NP NP	-Braun et al. Kidney int.2016 -ND

M, male; F, female; mat, maternal; pat, paternal; NP, not present; ND, not described; MOI, model of inheritance; AR, autosomal recessive; AD, autosomal dominant; XLR, X-linked recessive; XLD, X-linked dominant. Sequence variants nomenclature follows the last update of HGVS (den Dunnen et al. Hum Mutat. 2016).

Table 5. Clinical profile of patients with SRNS.

Group	Patient	Histology	Age at onset	Response to CNIs	Response to RASi	Remission	CKD stage at last follow-up	Post-transplant recurrence	Length of follow-up
Podocytopathies (21)	**Case 1**	FSGS	1 yr, 2 m	-	No	N	ESRD	No	90 m
	Case 2	FSGS	6 yr, 4 m	-	No	N	ESRD	No	72 m
	Case 3	FSGS	5 yr	No	No	N	ESRD	-	144 m
	Case 4	MCD	2 m	No	No	N	ESRD	-	160 m
	Case 5	FSGS	3 yr	No	Yes	P	ESRD	No	187 m
	Case 6	FSGS	2 days	-	No	N	ESRD	No	284 m
	Case 7	NP	4 m	-	No	N	ESRD	No	72m
	Case 8	FSGS	29 yr, 9 m	-	-	N	III	-	72 m
	Case 9	FSGS	11 yr, 3 m	No	No	N	I	-	48m
	Case 10	MCD	2 yr, 8 m	No	No	N	I	-	96 m
	Case 11	NP	4 yr	Yes	Yes	P	I	-	9m
	Case12	FSGS	6 yr	-	-	N	ESRD	No	132m
	Case 13	FSGS	13 yr	-	No	N	ESRD	No	96m
	Case 14	DMS	3 m	-	No	N	ESRD	No	180m
	Case 15	FSGS	7 yr, 7 m	No	No	N	ESRD	No	108m
	Case 16	FSGS	3 yr, 8 m	No	No	N	ESRD	No	94m
	Case 17	FSGS	7 yr, 5 m	No	Yes	P	ESRD	-	57m
	Case 18	FSGS	1 yr, 6 m	-	-	N	ESRD	No	25m
	Case 19	NP	1 yr	-	No	N	ESRD	No	30m
	Case 20	FSGS	8 yr, 6 m	-	-	N	III	-	7m
	Case 21	MCD	3 y, 9 m	No	No	P	I	-	24m

Phenocopies (17)	**Case 22**	FSGS	3 yr, 4 m	-	Yes	P	ESRD	-	88m
	Case 23	FSGS	26 yr, 2 m	-	Yes	P	III	-	12m
	Case 24	MCD	11 yr, 8 m	No	Yes	P	I	-	126m
	Case 25	FSGS	3 yr, 6 m	-	No	N	I	-	81m
	Case 26	MCD	4 y, 7 m	-	No	N	I	-	156m
	Case 27	FSGS	6 yr	-	0	N	ESRD	-	168m
	Case 28	FSGS	8 m	-	Yes	P	I	-	57m
	Case 29	FSGS	4 yr, 6 m	No	No	N	III	-	60m
	Case 30	FSGS	9 yr, 7 m	No	No	N	III	-	14m
	Case 31	FSGS	22 yr	-	Yes	P	III	-	17m
	Case 32	NP	9 yr	-	Yes	P	III	-	120m
	Case 33	FSGS	16 yr, 5 m	-	Yes	P	II	-	12m
	Case 34	FSGS	14 yr, 2 m	No	No	N	ESRD	-	100m
	Case 35	FSGS	1 yr, 6 m	-	No	N	II	-	81m
	Case 36	MCD	2 yr, 9 m	-	No	N	I	-	6m
	Case 37	FSGS	1 yr	No	No	N	II	-	89 m
	Case 38	MCD	5 yr	-	Yes	P	II	-	77 m

Negative (15)	**Case 39**	MCD	2 yr, 5 m	-	Yes	C	I	-	42m
	Case 40	FSGS	4 m	-	Yes	C	I	-	78m
	Case 41	FSGS	3 yr, 4 m	Yes	Yes	C	I	-	119m
	Case 42	FSGS	2 yr, 6 m	No	Yes	C	I	-	120m
	Case 43	FSGS	2 yr, 6 m	Yes	Yes	C	I	-	33,0
	Case 44	FSGS	2 yr, 8 m	-	Yes	P	I	-	27m
	Case 45	FSGS	2 yr, 7 m	Yes	-	C	I	-	28m
	Case 46	FSGS	2 yr, 6 m	No	No	P	II	-	143m
	Case 47	MCD	7 yr, 9 m	Yes	No	C	I	-	48m
	Case 48	MCD	5 yr	Yes	-	C	-	-	72m
	Case 49	MCD	5yr, 11 m	No	No	P	I	-	33m
	Case 50	FSGS	13 yr	No	No	N	I	-	18m
	Case 51	MCD	6 yr	No	No	P	I	-	52m
	Case 52	FSGS	6 yr	Yes	Yes	P	V	-	124m
	Case 53	MCD	13 yr	No	No	N	I	-	24m
Undefined (10)	**Case 54**	DMS	8 m	-	No	N	ESRD	No	96m
	Case 55	FSGS	2 yr	-	No	N	III	-	216m
	Case 56	FSGS	13 yr	No	No	N	ESRD	No	108m
	Case 57	FSGS	9 yr	No	No	N	III	-	125m
	Case 58	FSGS	3 yr, 5 m	No	No	N	ESRD	Yes	180m

	Case 59	FSGS	12 yr	No	No	N	ESRD	Yes	132m
	Case 60	MCD	5 yr, 10 m	No	No	N	ESRD	Yes	34m
	Case 61	FSGS	2 yr, 3 m	No	No	N	ESRD	Yes	149m
	Case 62	FSGS	29 yr, 6 m	-	-	N	ESRD	-	40m
	Case 63	FSGS	26 yr	-	-	N	ESRD	No	3m

FSGS, focal segmental glomerulosclerosis; MCD, minimal change disease; DMS, diffuse mesangial sclerosis; NP, not performed; yr, years; m, months; -, therapy not performed; C, complete remission; N, no remission; P, partial remission; CNIs, calcineurin inhibitors; RASi, inhibitors of the renin-angiotensin-aldosterone system; CKD, chronic kidney disease; ESRD, end stage renal disease.

Materials and Methods

Recruitment of patients and clinical investigation

In all the studies, patients were recruited only after the patient (whenever applicable), the parents or the legal guardians provided informed written consent. Moreover, all the patients enrolled were referred to Meyer Children's Hospital of Florence (Italy) for clinical evaluation and/or genetic testing.

In details, all the patients with a clinical diagnosis of dRTA (non–anion gap metabolic acidosis and the inability to maximally acidify the urine) and referred to Meyer Children's Hospital of Florence from 2011 to 2015 in need of a molecular diagnosis of the disease were included in this part of project. A total of 89 patients were recruited. All the patients underwent genetic testing with NGS target resequencing.

In addition, a total of 110 patients with a diagnosis of apparently sporadic idiopathic NS referred to Meyer Children's Hospital of Florence between 2000 and 2018 and aged 0-30 years were included in the study object of this part of the project. Patients were then sub-classified in SRNS or SSNS. In particular, steroid-resistance was defined as failure to achieve remission after 8 weeks of prednisone 60 mg/m^2/day or 2 mg/kg/day for 4 weeks followed by 40 mg/m^2 or 1.5 mg/kg on alternate days for 4 weeks, according to KDIGO guidelines (Glomerulonephritis Work Group: Improving Global Outcomes (KDIGO), 2012). Based on this definition, we selected 63 consecutive cases of SRNS, as well as 47 patients with the same clinical phenotype but who showed complete remission

of NS with an initial course of steroid therapy, according to KDIGO guidelines (Glomerulonephritis Work Group: Improving Global Outcomes (KDIGO), 2012). SSNS patients were selected among those in follow-up in our center because they showed a frequent-relapsing or steroid-dependent phenotype. None of the SSNS patients exhibited a phenotype of late-responder to steroid treatment. Among the 63 patients with SRNS, 11 were considered eligible for steroid treatment but were not treated for one of the following reasons: congenital NS, pathologic diagnosis of diffuse mesangial sclerosis, advanced renal failure at diagnosis. The latter were considered as comparable to SRNS, according to the literature (Glomerulonephritis Work Group: Improving Global Outcomes (KDIGO), 2012).

Exclusion criteria were: 1) incomplete clinical data set or unavailability of parents DNA; 2) poor DNA quality; 3) clinical, laboratory or biopsy signs of an immune-mediated disease and/or a secondary form of NS according to KDIGO guidelines (Glomerulonephritis Work Group: Improving Global Outcomes (KDIGO), 2012); 4) prevalent tubular proteinuria; 5) evident extra-renal signs/symptoms of another disorder or syndromic SRNS; 6) known familiarity. All SRNS patients were checked at diagnosis for the presence of SNHL and ocular abnormalities.

Throughout all the parts of the research project, all information about demographic, clinical features, disease onset, response to treatment, histologic findings and final diagnosis including follow-up was collected retrospectively from direct interview of the patient and medical records. Complete, partial and

no remission were defined according to KDIGO guidelines (Glomerulonephritis Work Group: Improving Global Outcomes (KDIGO), 2012). Estimated glomerular filtration rate (eGFR) was calculated using Schwartz equation or MDRD equation, owing to the age of patients. Indeed, eGFR was used to assess the presence of CKD. CKD was defined as eGFR < 90/ml/min *per* 1.73 m^2 of body surface area. ESRD was defined as eGFR < 5/ml/min *per* 1.73 m^2 of body surface area or as the requirement of renal replacement therapy.

Parents and/or relatives were either included prior to this study or requested to participate after the identification of potentially causative variants in the patient. The local Ethics Committee of the Meyer Children's Hospital of Florence approved the study.

Sequencing

Peripheral blood DNA was extracted using QIAamp Mini Kit (QIAGEN®, Hilden, Germany), according to manufacturer's instructions, and quantified by NanoDROP 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

To analyze sequence variations in *SLC4A1* (NM_000342), *ATP6V0A4* (NM_020632), and *ATP6V1B1* (NM_001692) genes, we used a strategy based on the locus-specific amplification of genomic DNA for each amplicon separately, followed by Roche 454 sequencing. Fusion primers were designed to generate tiled amplicons ranging in size from 300- to 400-bp segments. At the 50 end, fusion primers contained a MID sequence that is a nucleotide tag that identifies

the different samples. The MID was selected from a list provided by Roche (Palazzo V, 2017).

To construct DNA libraries for WES, we used a strategy based on enzymatic fragmentation to produce dsDNA fragments followed by End repair, A-tailing, adapter ligation and library amplification (Kapa Biosystems, Wilmington, MA). Libraries were hybridized with the protocol SeqCap EZ Exome v3 (Nimblegen, Roche, Basel, Switzerland), and sequenced with NextSeq500 (Illumina Inc., San Diego, CA).

Assembly, Variant Calling

The reads were aligned with the human reference hg19 genome using Burrows-Wheeler Aligner (BWA), mapped and analyzed with the IGV software (Integrative Genome Viewer, 2013 Broad Institute). The variant call for identification of nucleotide variants was performed using automated pipelines (Genome Analysis ToolKit Unified Genotyper Module, GATK).

Variant interpretation strategy for target resequencing and WES

In order to prioritize genetic variants with a possible pathogenic significance after target resequencing, we used the algorithm previously described (Giglio S, 2015). Variants already described were considered as pathogenic, in accordance with the pattern of inheritance. *In silico* analysis was performed using Polyphen-2 (http://genetics.bwh. harvard.edu/pph2/), SIFT (http://sift.jcvi.org/), Mutation Taster (http:// www.mutationtaster.org/), or

BDGP splice site prediction (http://www. fruitfly.org/seq_tools/splice.html) prediction tools.

Regarding WES, variants were filtered *in silico* for a panel of 284 genes (Table 3) known to be associated with CKD. A team of nephrologists, clinician scientists and geneticists performed variants prioritization.

Variants were classified in agreement with the interpretation guidelines of the ACMG (Richards S, 2015).

In details, we selected only non-synonymous, short insertion/deletion or splice-site variants (30 bp splice acceptor, 30 bp splice donor) with the following characteristics (Figure 7):

-Variants not present or with a minor allele frequency ≤ 0.01 for AR- and with a minor allele frequency ≤ 0.001 for AD-transmitted genes in population database "1000 Genomes Project", "Exome Variant Server", dbSNP147, ExAC, gnomAD. We used manual inspection for the p.[Arg229Gln] variant in *NPHS2* when segregating with variants localized in exon 7 and 8 and for *APOL1* G1and G2 variants.

-Variants not present or with a minor allele frequency ≤ 0.01 for autosomal recessive (AR) and with a minor allele frequency ≤ 0.001 for autosomal dominant (AD)-transmitted genes in "in-house" exome control cohort (300 exomes) of unrelated subjects analyzed for non-renal diseases or variants.

-Variants predicted as damaging by at least 4 over 6 *in silico* tools (Polyphen-2, SIFT, Mutation Taster, Mutation Assessor, FATHMM, FATHMM MKL). Variants predicted to be damaging by less than 4 *in silico* tools were taken into

account when they represented the second mutated allele in presence of a potentially pathogenic variant and were consistent with the clinical phenotype.

-Variants correctly segregating within the family or representing *de novo* variants.

Variants were classified as variants of unknown clinical significance when they were predicted as damaging by less than 4 *in silico* tools or did not segregate within the family.

Variants were classified as potentially pathogenic if they were consistent with the clinical phenotype of the patient and family carriers after reverse phenotyping (Figure 7). If no potentially pathogenic variant was identified filtering for 284 genes associated with chronic kidney disease, we extended the analysis to all the exome.

All the variants identified by target resequencing or WES were validated by Sanger sequencing.

a-CGH

a-CGH was performed using a custom Agilent Human Genome CGH Microarray (Agilent Technologies, Santa Clara, CA). We used the same protocol as previously described.31 This platform is a resolution of 10 kbp in the regions of interest. Text output from the quantitative analyses were imported into Genomic Workbench Standard Edition 5.0 software (Agilent Technologies).

Urine-derived renal progenitor cells cultures and differentiation

Fresh urine samples from the patient affected by LN and from controls were prepared as previously described (Lazzeri E R. E., 2015). Informed written consent from parents or legal guardians has been obtained before sampling. The group of controls comprised patients affected by different glomerular diseases, including LN, who responded to treatment and who did not carry any potentially pathogenic variants in the genes included in the panel used for target resequencing (Table 1). Patient-specific u-RPC cultures were maintained as described (Lazzeri E R. E., 2015). Podocyte differentiation was obtained by exposing u-RPC to the differentiation medium VRAD, composed by DMEM/F12 (Sigma-Aldrich, St. Louis, MO, USA) supplemented by 10% fetal bovine serum (Hyclone Laboratories, South Logan, UT), 100 nM vitamin D and 100 μM retinoic acid, for 48 hours (all Sigma-Aldrich).

Flow cytometry analysis of cell viability by Annexin V/Propidium Iodide uptake

Cellular necrosis/apoptosis was evaluated by flow cytometry following the staining for Annexin V and Propidium Iodide (BD Biosciences, San Diego, CA, USA). Flow cytometry was performed using the software FACSDiva.

Immunofluorescence and confocal microscopy

Confocal microscopy was performed on cells cultured on chamber slides as described using the confocal laser microscope Leica TCS SP5-II (Leica, Milan,

Italy) as previously described (Lazzeri E R. E., 2015). We used monoclonal antibody anti-nefrin clone E-1 (Santa Cruz Biotechnology, Dallas, Texas, USA) for the staining of the protein nefrin. The secondary antibody was Alexa Fluor goat anti-mouse IgG2b 488 (Invitrogen, Life Technologies, Monza, Italy). Cytoscheleton structure was evaluated by the staining of cells with the marker Phalloidin Alexa Fluor 488 (Molecular Probes from Invitrogen). We used the nuclear marker DAPI to counterstain nuclei in all the experiments. Lysosomal staining has been obtained with the lysosome marker Lysotracker (Molecular Probes from Invitrogen), according to the manufacturer instructions.

Quantification of the mean fluorescence intensity of the lysosomal marker Lysotracker

The mean intensity of fluorescence of the lysosomal marker Lysotracker has been measured in the region of interest (ROI) traced by the analysis software Leica Las AF. We considered as ROI the surface area of the cell. The analysis was performed by evaluating cells in 10 different fields in 3 separate experiments by two independent operators. The results are expressed as mean ± standard error.

Statistical analysis

Statistical analysis was performed using SPSS statistical software (SPSS, Inc., Evanston, IL). All continuous data are expressed as mean ± standard error and range, unless otherwise stated. Between-group comparisons were performed

using one-way ANOVA for normally distributed variables. *Post hoc* analysis was performed according to the Bonferroni's correction for multiple comparisons. Frequencies among groups were compared by chi-squared test with Fisher's correction, Student *t* test and Mann-Whitney test, according to the type of variable considered.

Kaplan-Meier estimates were used to generate an overall survival curve for the development of ESRD and differences among groups were assessed by log-rank test.

A P value <0.05 was considered statistically significant.

Contribution of the applicant

I had been responsible for patients recruitment and enrollment and I handled clinical evaluation and collection of clinical and laboratory information. Moreover, I handled urine-derived renal progenitor cells cultures and differentiation and performed immunofluorescence and confocal microscopy on RPC and podocytes cultures, in collaboration with co-workers with expertise in these techniques. Finally, I had been responsible for statistical analysis and manuscript preparation.

My co-workers performed the other parts of the project.

Discussion

NGS technology undoubtedly prompted a significant impulse to the discovery of the genetic basis of inherited kidney diseases. Indeed, the wide availability of NGS led a spreading of its application in the diagnosis of genetic kidney disorders, allowing us to better identify the genetic background of a significant proportion of them. Moreover, the decrease in economic costs and the shortening of the time required to obtain the results of sequencing made genetic testing more suitable for daily clinical practice. Notwithstanding this, NGS raises questions that are still waiting to be answered. NGS, and in particular WES, results in a high number of genetic variants in each single run of sequencing. Even following a bioinformatic filtering for variants selection, it is likely that a not negligible number could be classified as variants of unknown clinical significance. The assessment of role of these variants is difficult and requires clinical and scientific efforts. Indeed, an accurate phenotyping, probably also in the setting of reverse phenotyping, is unavoidable to address this issue. In addition, although NGS has been improving the diagnosis of many inherited kidney diseases, the molecular basis of a significant proportion of them still need to be identified.

In this research project we showed how the application of NGS could result in the construction of a personalized management of patients affected by inherited nephropathies. Indeed, the results of this study demonstrate that:

1. NGS is helpful in identifying the genetic etiology of inherited nephropathies, including SRNS and dRTA. A correct and reliable identification of the etiology of inherited nephropathies is essential for a critical revision of their classification. High-throughput genetic testing unequivocally represents an important instrument to this aim, especially if the results of genetic screening are consistently correlated with clinical features. In this context, reverse phenotyping, that is to say a careful clinical re-evaluation of the patient and his/her family on the basis of genetic findings, could be critical. Anyway, many genetic causes of inherited nephropathies still remain to be identified (according to results of this project, about 30% of patients with SRNS and dRTA do not carry any potentially pathogenic variant). This could have different explanation, including the presence of not yet identified genes that are therefore not included in the gene panels analyzed (either by target resequencing or by bioinformatic filtering). Besides this, thus far the role of so-called modifier genes (e.g. genes that are not directly responsible for the disease but that could act in modifying the phenotype) or modifier variants (e.g. heterozygous variants in AR-transmitted genes, even in a digenic pattern of inheritance), as well as of epigenetic factors is unexplored. Genetic variants are defined as variants of unknown clinical significance if they not fulfill the criteria for pathogenicity. Their clinical relevance is difficult to be established, especially in the absence of a reliable family pedigree, but it is possible that their contribute in at

least some clinical features could be not negligible, as suggested by the rapid progression toward ERDS of patients affected by SRNS and classified as undefined in this project or by the similar phenotype of patients affected by dRTA and classified as carrying variants of unknown clinical significance to that of patients with pathogenic mutations. Clarifying the role of these factors would finally lead a much deeper understanding of the pathophysiology of many renal diseases. Nowadays, this body of work offers insights into possible mechanism of kidney disease and its complications, but as yet the field has not matured sufficiently that it can make only few recommendations that will affect the practice of medicine (Kopp JB W. C., 2018).

2. The results of genetic screening are critical for an adequate planning of therapeutic strategy. This is particularly important for SRNS. Indeed, patients who result to be affected by a genetic disease (either podocytopathies or phenocopies) should not receive IS therapy since it is not only unhelpful but even potentially detrimental. Moreover, the results of this project suggest that IS resistance, if not otherwise explained, should prompt genetic testing also in cases of secondary forms of SRNS, like LN (Romagnani P G. S., 2016). Genetic testing could be of help also in treating patients affected by dRTA since the need for alkali and potassium supplementation is usually milder in patients with mutations in *SCL4A1* in comparison with patients with mutations in *ATP6V0A4* and *ATP6V1B1*.

3. An accurate genetic diagnosis could stratify the risk of CKD progression and define patient prognosis. Since the pathophysiologic mechanisms of SRNS are still poorly understood, defining patient prognosis and the risk of CKD progression is hard. Indeed, the role of classical risk factors for CKD progression in children is a matter of debate. The results of this project highlight the critical relevance of genetics in outcome determination. Indeed, children affected by SRNS due to monogenic podocytopathies are known to progress to ESRD usually in their infancy or adolescence. This could also be the case of patients with secondary form of SRNS (e.g. LN) who anyway do not respond to IS therapy (Romagnani P G. S., 2016). In this study we demonstrated that among young patients affected by SRNS currently considered as not genetic a significant proportion carry pathogenic mutation in genes other the podocytes genes and can be defined as phenocopies of podocitopathies. These patients show a significantly better outcome in comparison to patients with podocitopathies, with a slower progression of CKD and a lower incidence of ESRD at 10 years from the disease onset. Quite surprisingly, CKD can occur at a frequency higher than expected in patients with dRTA (Palazzo V, 2017). The reasons for this have yet to be clarified but it is possible to hypothesize that the genetic background, together with others, could be one of the influencing factors.
4. Genetic testing allows clinician to provide patients and their family with a reliable genetic counseling.

In conclusion, the results of this project place themselves in the context of the so-called personalized or precision medicine for inherited nephropathies. To address patients' benefit, the recent advances in the genetics of kidney diseases and CKD need to be inserted in a strategy for future development. After securely identifying causal genetic variants, disease modeling is necessary to understand the impact each variant has on relevant pathways, the mechanisms of the disease and how they are perturbed by genetic abnormalities. This would finally lead to identify therapeutic approaches that will ideally return these pathways to physiologic function, or at least to counter harmful effects (Kopp JB W. C., 2018). Afterwards, this knowledge could prompt to carefully design clinical trials dedicated to inherited nephropathies. Moreover, this information could allow us to understand whether genetic results, including information on CKD progression, would alter patient management (e.g. selection of therapy, extent of patient counseling). By integrating genetic findings with basic research and clinical evaluation, we will probably be able to provide patients affected by inherited nephropathies with a really personalized approach capable of improving patient outcomes with regard to CKD progression and/or survival and quality of life.

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List of abbreviations

ACMG, American College of Medical Genetics and Genomics

AD, autosomal dominant

aHUS, atypical heamolitic uremic syndrome

AR, autosomal recessive

ARPKD, autosomal recessive polycystic kidney disease

CAKUT, congenital abnormalities of the kidney and urinary tract

CKD, chronic kidney disease

dRTA, distal renal tubular acidosis

eGFR, estimated glomerular filtration rate

ESRD, end-stage renal disease

FSGS, focal segmental glomerulosclerosis

FTT, failure to thrive

GBM, glomerular basement membrane

GBMP, GBMopathies

GWAS, genome-wide association studies

IS, immunosuppressive

KDIGO, Kidney Disease: Improving Global Outcomes

LN, lupus nephritis

MCD, minimal change disease

MMF, micophenolate mofetil

NGS, next-generation sequencing

NS, nephrotic syndrome

RASi, renin-angiotensin-aldosterone system

RLN, refractory lupus nephritis

ROI, region of interest

SNHL, sensorineural hearing loss

SSNS, steroid-sensitive nephrotic syndrome

SRNS, steroid-resistant nephrotic syndrome

u-RPC, urine-derived renal progenitor cells

WES, whole-exome sequencing