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GENOMIC STUDY OF EARLY-ONSET CHRONIC KIDNEY DISEASE

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Genomic study of early-onset chronic kidney disease

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ABBREVIATIONS

aCGH	Array comparative genomic hybridization
ACMG	American College of Medical Genetics and Genomics
AD	Autosomal dominant
ADAS	Autosomal dominant Alport syndrome
ADPKD	Autosomal dominant polycystic kidney disease
ADTKD	Autosomal dominant tubulointerstitial kidney disease
AMP	Association for Molecular Pathology
AR	Autosomal recessive
ARAS	Autosomal recessive Alport syndrome
ARPKD	Autosomal recessive polycystic kidney disease
CAKUT	Congenital anomalies of the kidney and urinary tract
CKD	Chronic kidney disease
ClinGen	Clinical Genome Resource
CMA	Chromosomal microarray analysis
CNV	Copy number variant
DNA	Deoxyribonucleic acid
FSGS	Focal segmental glomerulosclerosis
GFR	Glomerular filtration rate
GAMOS	Galloway-Mowat syndrome
gnomAD	Genome Aggregation Database
GWAS	Genome-wide association analysis
HGMD	Human Gene Mutation Database
IgAN	IgA nephropathy
IKD	Inherited kidney diseases
INDELs	Small insertion-deletions
IPNA	International Pediatric Nephrology Association
KF	Kidney failure
KRT	Kidney replacement therapy
LOVD	Leiden Open Variation Database
LCRs	Low copy number repeats
MODY	Maturity-onset diabetes of the young
MLPA	Multiplex ligation-dependent probe amplification
MN	Membranous nephropathy
NGS	Next generation sequencing

NIH	National Institute of Health
NPHP-RC	Nephronophthisis and related ciliopathies
NS	Nephrotic Syndrome
PCR	Polymerase chain reaction
Pmarp	Per million of age-related population
RD	Related disease
SNPs	Single nucleotide polymorphisms
SNVs	Single nucleotide variants
SRNS	Steroid-Resistant Nephrotic Syndrome
TSC	Tuberous sclerosis complex
UAlb/Cr	Urinary albumin/creatinine ratio
UProt/Cr	Urinary protein/creatinine ratio
VCEPs	Variant Curation Expert Panels
WES	Whole exome sequencing
WGS	Whole genome sequencing
XLAS	X-linked Alport syndrome

INTRODUCTION

1. CHRONIC KIDNEY DISEASE (CKD)

Chronic kidney disease (CKD) is defined as abnormalities of the kidney structure or function, present for more than 3 months (Inker *et al.*, 2014). It includes albuminuria, urine sediment abnormalities, electrolyte and other abnormalities due to tubular disorders, abnormalities detected by histology, structural abnormalities detected by imaging, history of kidney transplantation, or a decreased glomerular filtration rate (GFR) of <60ml/min/1.73m2. Nevertheless, normal GFR in children under two years of age is less than 60 ml/min/1.73 m2, and it is not until approximately 2 years of age that body surface area adjusted GFR values comparable to those seen in the adult (Hogg *et al.*, 2003).

The severity of CKD has been classified into 5 categories or stages based on GFR and 3 categories of albuminuria (Figure 1). This is because proteinuria stands out as the most powerful modifiable prognostic factor of CKD progression. Kidney failure (KF) represents the final stage of CKD culminating in the need for kidney replacement therapy (KRT), in the form of either renal transplantation or dialysis.

KDIGO 2012			Albuminuria Categories, description and ranges		
			A1	A2	A3
			Normal to milddly increased	Moderately increased	Severely increased
Glomerular filtration rate Categories, description and ranges (ml/min/1,73 m²)			< 30 mg/gª	30-300 mg/gª	> 300 mg/gª
G1	Normal or increased	≥90			
G2	Milddly decreased	60-89			
G3a	Milddly to moderately decreased	45-59			
G3b	Moderately to severely decreased	30-44			
G4	Severely decreased	15-29			
G5	Renal failure	<15			

Figure 1. Prognosis of CKD according to the glomerular filtration rate and albuminuria categories. Green: low risk; Yellow: moderately increased risk; Orange: high risk; Red, very high risk. (Inker *et al.*, 2014).

CKD is a major health problem, affecting more than 10% of people worldwide, with substantial associated morbidity and mortality. Epidemiologic data on CKD may underestimate its real incidence and prevalence since CKD is often clinically asymptomatic, especially in earlier stages. In addition, most of the epidemiologic information originates from data available on KF, when treatment becomes necessary to sustain life. Despite these, the paediatric incidence of CKD in Europe 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 (Ardissino *et al.*, 2003; Harambat *et al.*, 2012).

The aetiology of CKD in children and young adults differs significantly from that in the elderly population. Inherited kidney diseases (IKD) are the leading cause of CKD in children. The main clinical diagnostic groups of early-onset CKD, defined as CKD manifesting in the first three decades of life, include congenital anomalies of the kidney and urinary tract (CAKUT) (~50% of cases), glomerulopathies (~20% of cases), cystic kidney diseases (~6–10% of cases), and tubulopathies (~2% of cases)(*North American Pediatric Renal Trials and Collaborative Studies: NAPRTCS Annual Transplant Report*, 2014; Vivante and Hildebrandt, 2016). Advancing knowledge of etiologic causes of CKD is paramount for patients with CKD in order to better understand the pathogenesis, have an adequate classification, prognosis, and personalized medicine approach.

2. GENETIC CAUSES OF CKD

2.1. MONOGENIC CAUSES

Monogenic kidney diseases may be classified based on the mode of inheritance into autosomal dominant (AD), autosomal recessive (AR), and X-linked.

In AD disease, disease-causing variants in one of the two parental alleles are sufficient to cause the disease. Therefore AD diseases tend to segregate through multiple generations in a single family. AD disease-causing genes often present features such as variable expressivity and incomplete penetrance, resulting in a weakened genotype–phenotype correlation. There is the same risk for both sexes to develop or transmit the disease and each offspring has a 50% chance of inheriting the affected allele.

In AR disease, disease-causing variants in both alleles are needed for the disease to manifest. The patient usually has unaffected parents who are heterozygous carriers of a disease-causing variant in the same gene. The risk of disease recurrence in each sibling of the proband is 25%, and it affects males and females equally. The disease usually manifests earlier in life than AD diseases, and presents full penetrance. Parental consanguinity increases the chances that a couple will both carry the same disease-causing variant and hence it is associated with an increased birth prevalence of recessive monogenic disorders.

X-linked diseases generally are more severe in males than in females. This is due to the fact that males are hemizygous for the genes of the X chromosome (they only have one copy) and females (with two X chromosomes) present the phenomenon of X chromosome inactivation during the preimplantation development. The inactive X can be paternal or maternal and is inactivated randomly but permanently. Due to unknown reasons there may be a skewed

inactivation towards either the wild type allele or the mutated one. This phenomenon explains the variability of the severity of the disease in females. All daughters of an affected male will be heterozygous for the disease-causing variant and there is no male-to-male transmission of the disease. Affected homozygous females are exceptionally rare.

Monogenic diseases are an underestimated, but very important cause of CKD. Approximately 25% of patients with CKD report a family history, revealing the strong role of genetics in kidney disease (Connaughton *et al.*, 2015). Monogenic causes are estimated to account at least for 10% of unselected adults with CKD (Groopman *et al.*, 2018) and in >30% of children with kidney disease (Vivante and Hildebrandt, 2016; Mann *et al.*, 2019). Monogenic causes of CKD encompass a broad range of different CKD phenotypes and genetic analysis is therefore essential for establishing precise diagnoses in these patients. Currently, more than 400 disease genes causative or associated with kidney disease, and the list keeps on expanding.

2.1.1. CAKUT

CAKUT constitute the most common cause of early-onset CKD, affecting three to six of 1000 live births, and is a significant contributor to CKD in adults (*North American Pediatric Renal Trials and Collaborative Studies: NAPRTCS Annual Transplant Report*, 2014; Chesnaye *et al.*, 2014). Data from European kidney registries show that it is the main cause of KF in the paediatric age, representing 41.3% of patients receiving KRT (Chesnaye *et al.*, 2014) with a mean age of KF of 31 years old versus 61 years in patients with other kidney diseases (Wühl *et al.*, 2013).

CAKUT collectively refers to a diverse group of structural malformations that result from perturbations in embryonic kidney and urinary tract development. These abnormalities can alter the upper urinary tract (renal agenesis, renal hypoplasia, renal dysplasia, ectopic or horseshoe kidney) and / or the lower urinary tract (vesicoureteral reflux, obstructive uropathy, posterior urethral valves, duplication of the collecting system) (Figure 2). Additionally, some anomalies are often concurrent, such as vesicoureteral reflux and duplex collecting system (Pope IV *et al.*, 1999). CAKUT is characterized by high variable clinical course, which in the most severe bilateral cases can lead to perinatal death or the need of premature dialysis. In contrast, some patients with CAKUT are asymptomatic during childhood but can develop CKD in adulthood (Wühl *et al.*, 2013). CAKUT can present as isolated or syndromic, associated with various extrarenal phenotypes.

The existence of syndromic phenotypes, familial clustering, and more than 180 monogenic mice models with CAKUT, suggest a major genetic contribution to the aetiology of CAKUT and a considerable monogenic cause. In recent years, alterations in more than 50 genes have been shown to cause isolated or syndromic CAKUT, in AD or, less frequently, AR mode of inheritance. Most of these genes code for key transcription factors or signaling molecules in embryonic development of the kidney and urinary tract, and have incomplete penetrance and variable expressivity. Disease-causing variants in these genes, however, only explain 10% to 20% of CAKUT cases (Capone *et al.*, 2017). In addition, in the majority of CAKUT with an identified monogenic cause there is variable expressivity, and identical pathogenic variation can result in different CAKUT subphenotypes and in extremely variable severity, even within the same family.
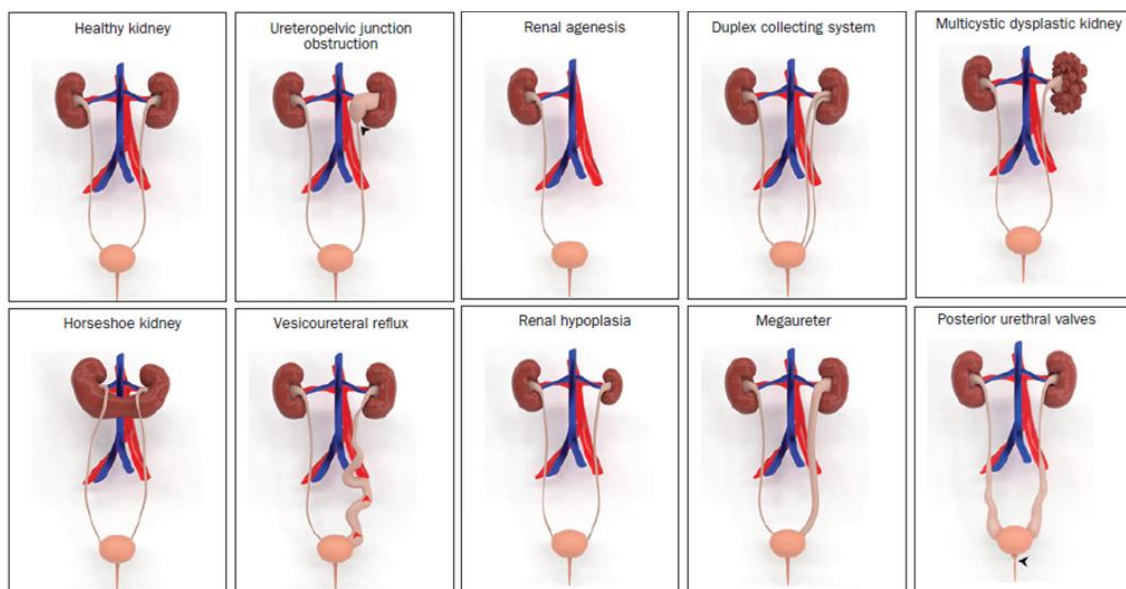
Figure 2. Overview of CAKUT phenotypes. The black arrows indicate the site of obstruction in ureteropelvic junction obstruction and posterior urethral valves. The red arrows indicate the abnormal flow of the urine from the bladder to the ureter or kidney occurring in vesicoureteral reflux. Figure from (Nicolaou *et al.*, 2015).

Currently, *HNF1B* and *PAX2* are the primary genes screened for disease-causing variants in patients with CAKUT and follow an AD pattern. Many studies confirmed that disease-causing variants in these two genes can explain up to 15% cases of CAKUT (Weber *et al.*, 2006; Thomas *et al.*, 2011; Madariaga *et al.*, 2013), making them the most important genes to screen for diagnostic purposes. Disease-causing variants in *PAX2* seem to be more frequently associated with renal hypodysplasia, while in *HNF1B* are more frequently associated with renal dysplasia (Weber *et al.*, 2006; Thomas *et al.*, 2011; Madariaga *et al.*, 2013). Around 40-60% of patients with disease-causing variants in *HNF1B* carry a *de novo* variant, and about half of the described

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variants consists of whole heterozygous gene deletions (Bellanné-Chantelot *et al.*, 2005; Heidet *et al.*, 2010).

In large studies, the genetic diagnostic rate identified in patients with CAKUT ranges from 1 to 20% depending on study inclusion criteria and methods of analyses, suggesting that most cases of CAKUT have not a monogenic aetiology. For instance, a targeted next generation sequencing (NGS) panel of 208 genes was sequenced in a heterogeneous cohort of 453 CAKUT patients, many of whom presented mild forms of unilateral CAKUT, identified the causative disease-causing variant just in 1.3% of cases (Nicolaou *et al.*, 2016). Otherwise, another study sequenced a panel of 330 genes in 204 CAKUT patients, 45% of whom presented severe forms of CAKUT, and identified the causative disease-causing variant in 18% of the cases (Heidet *et al.*, 2017). Whole exome sequencing (WES) has been used in a selected cohort of 232 families with CAKUT (40% consanguineous, 7% CAKUT syndromic, 3% CAKUT very severe, 17% familial cases) in which mutations in the *HNF1B, PAX2, EYA1, GATA3, SIX1,* and *SIX5* genes had been previously ruled out. In this study, a monogenic cause was identified in 14% of the families and new candidate genes for monogenic CAKUT were reported in 8% (19/232) (Van Der Ven *et al.*, 2018). Several studies have also demonstrated that copy-number variants (CNVs) contributed to the genetic architecture of CAKUT, with up to 16.6% of patients with renal hypodysplasia having a molecular diagnosis attributable to CNVs (Sanna-cherchi *et al.*, 2012). Another study reported a genome-wide association analysis (GWAS) of CNV in 2,824 CAKUT cases and 21,498 controls. They identified that the cases had a significant exonic CNV load and were enriched for known genomic disorders, especially for certain CAKUT phenotypes (renal agenesis, hypoplasia/dysplasia, posterior urethral valves). They detected CNVs in 4% (112/2,824) of patients with CAKUT, 65% (73/112) of which had CNVs in 6 loci: 17q12, where the *HNF1B* gene is located (23%); 22q11.2, corresponding to DiGeorge syndrome (15%); 16p11.2 (8%); 16p13.11 (8%); 1q21.1 (6%) and 4p16.1-p16.3 corresponding to Wolf-Hirschhorn syndrome (5%) (Verbitsky *et al.*, 2019). Moreover, the clinical phenotype and severity of CAKUT can vary markedly among patients, both within and between families with the same underlying disease-causing variant, demonstrating the complex genotype-phenotype relationship in CAKUT. In addition, monogenic CAKUT genes frequently have dominant inheritance with incomplete penetrance, such as *HNF1B* and *PAX2* genes.

Notably, the large majority of CAKUT cases are currently not explained by pathogenic variants in known or in novel identified genes. This might be due to disease-causing variants difficult to detect by NGS, to the involvement of somatic events, environmental factors or epigenetic

mechanisms, and to oligogenicity, which may explain both the known familial aggregation of CAKUT and the low rate of disease-causing variants identified in genes involved in monogenic forms of the disease (Nicolaou *et al.*, 2015). Last but not least, although CAKUT is a congenital condition it does not mean that there is a genetic cause behind each case. Non-genetic abnormalities during the process of embryogenesis may account for a significant percentage of negative genetic testing among CAKUT patients.

2.1.2. Cystic kidney diseases

Cystic kidney diseases encompass a broad spectrum of diseases and syndromes characterized by the formation of multiple renal cysts that alter the structure of the nephron. Most of the causative genes are localized in the "primary cilia," which are immotile sensory organelles that occur singly on the cell surface, and mutations of these genes give rise to deficient function of the cilia, with consequent formation of cysts. These nephropathies can manifest with a wide range of phenotypes, ranging from late onset mild forms to very severe forms with perinatal death. The distribution of cystic IKD according to the age at diagnosis is shown in Figure 3. To date, about 100 genes have been described as causative of monogenic cystic kidney diseases.

Autosomal dominant polycystic kidney disease (ADPKD) is the most common IKD; usually manifesting in adulthood and representing 6–10% of the population in KRT. It is a multisystemic disorder characterized by the development and inexorable expansion of multiple cysts scattered throughout the parenchyma of both kidneys. Progressive loss of kidney function takes place over many decades and frequently leads to KF during or after the sixth decade of life. Affected individuals might exhibit extrarenal manifestations, including hepatic and pancreatic cysts, intracranial aneurysms, abdominal hernias, and cardiac valvular lesions. It is caused by disease-causing variants in two major genes *PKD1* (78% cases), *PKD2* (15%), and rarely by variants in the recently described genes *GANAB* , *DNAJB11*, and *ALG9* (Cornec-Le Gall *et al.*, 2018; Cornec-Le Gall, Torres and Harris, 2018; Besse *et al.*, 2019). However, in 7–10% of patients with ADPKD, the causal variant is not identified (Bergmann *et al.*, 2018). Genotype-phenotype correlation studies have demonstrated that the gene and the type of causative variant are key factors to explain much of the clinical variability in ADPKD. Patients with-causative variants in *PKD2* have a more favourable renal prognosis (with median age at KF of around 79 years) than patients with causative variants in *PKD1* (with median age at KF of around 58 years) (Magistroni *et al.*, 2003; Cornec-Le Gall *et al.*, 2013, 2016; Hwang *et al.*, 2016). Disease-causing variants in *GANAB* are typically associated with mild ADPKD and preserved kidney function, and in *DNAJB11* have been described in few patients with normal

or small-sized kidneys with multiple small renal cysts with possible evolution to KF after 60 years of age (Besse *et al.*, 2017; Cornec-Le Gall *et al.*, 2018). In addition, patients with *PKD1* disease-causing variants that predicted to truncate the protein are associated with worse renal prognosis than those with non-truncating variants (Cornec-Le Gall *et al.*, 2013).
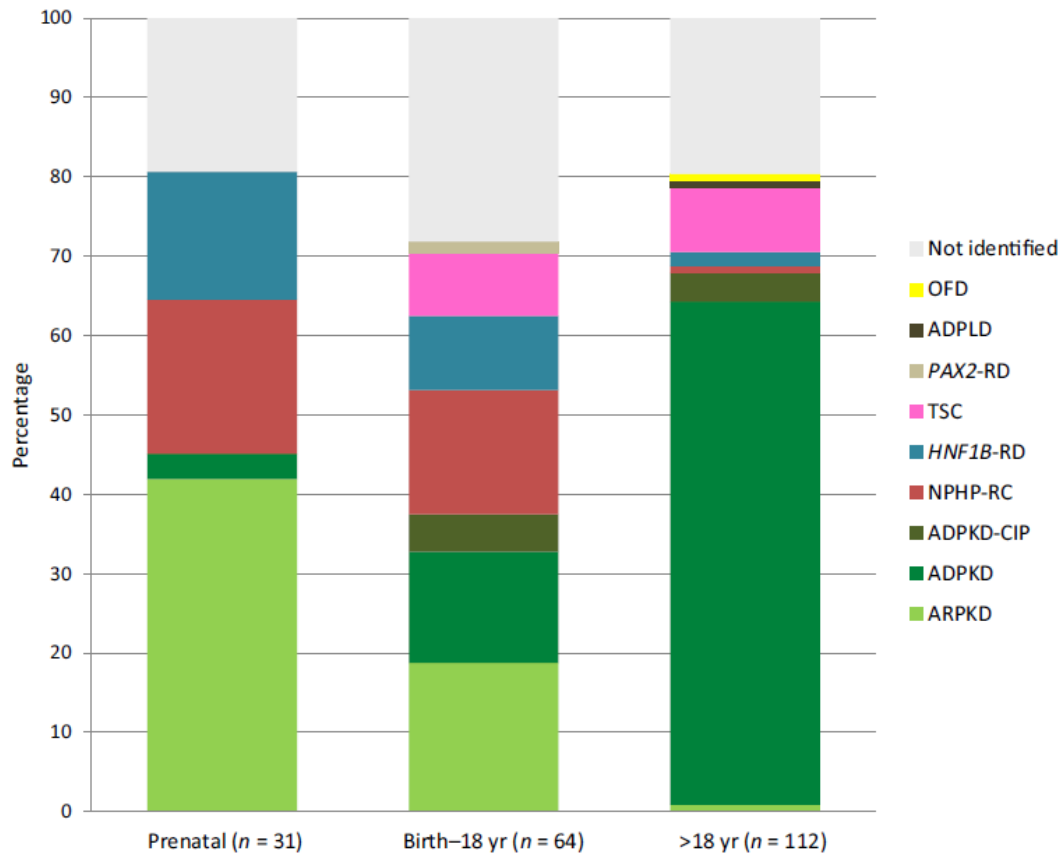
Figure 3. Distribution of cystic inherited kidney diseases according to the age at diagnosis. ADPKD, autosomal dominant polycystic kidney disease; ADPLD, autosomal dominant polycystic liver disease; ARPKD, autosomal recessive polycystic kidney disease; CIP, complex inheritance patterns; NPHP-RC, nephronophthisis-related ciliopathies; OFD, oral-facial-digital syndrome; RD, related disease; TSC, tuberous sclerosis complex. Figure from (Bullich *et al.*, 2018).

Autosomal recessive polycystic kidney disease (ARPKD) occurs in about one in 20,000 live births, and generally manifests in the perinatal period. Typically, the disease arises in the late gestational or neonatal stage, manifesting with the "Potter" phenotype (referred to a group of features associated with a lack of amniotic fluid and KF in an unborn infant) with massively enlarged kidneys, pulmonary hypoplasia, and characteristic facies. Of the affected infants, ~30% are reported to succumb to the disease, primarily due to respiratory compromise. One-year survival rates have been improved by advances in supportive therapy, dialysis, and transplantation, being of 92–95% in patients who survive the first month of life (Hartung and Guay-Woodford, 2014).
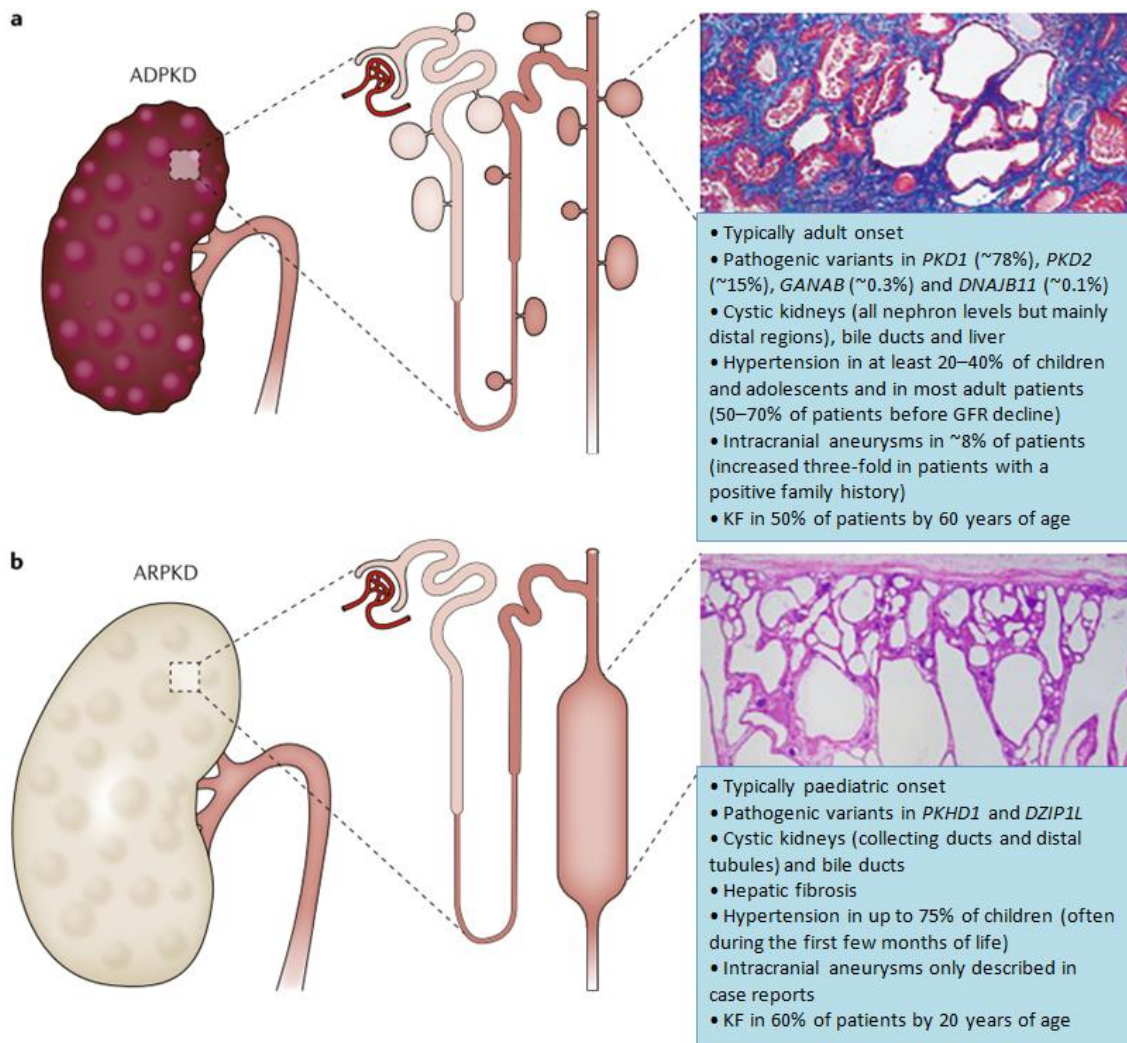
Figure 4. Renal and extrarenal manifestations in patients with ADPKD and ARPKD. Respective dilated collecting ducts run perpendicular to the renal capsule (renal section is stained with haematoxylin and eosin). Adapted from (Bergmann, 2018).

However, the spectrum for phenotypic severity might be much broader than widely assumed with some elderly reported patients with ARPKD who were only moderately affected (Bergmann *et al.*, 2005). ARPKD is mainly caused by disease-causing variants in the *PKHD1* gene and rarely in the *DZIP1L* gene (Lu *et al.*, 2017). However, the clinical characteristics of ARPKD can be mimicked by disease-causing variants in a number of other cystic genes (Bergmann, 2018). Genotype-phenotype correlations for *PKHD1* have been established for the type of variant. Typically patients with 2 truncating variants are severely affected and display peri- or neonatal mortality. Patients with at least 1 missense variant tend to be less severely affected and are more likely to survive the neonatal period. However, as to be expected, some missense changes affect critical residues and can clinically impress as severe as truncating variants (Bergmann *et al.*, 2005). About 2–5% of patients with ADPKD show an early and severe phenotype clinically indistinguishable from ARPKD (Bergmann, 2015). Some of these

severely affected patients carry disease-causing variants in more than one cystic gene or a hypomorphic *PKD1* variant *in trans* with a *PKD1* causative variant, probably aggravating the phenotype (Bergmann *et al.*, 2011).

Nephronophthisis and related ciliopathies (NPHP-RC) includes a broad range of paediatric AR diseases with high genetic heterogeneity characterized by corticomedullary cysts and small or normal size kidneys. About 10–15% of NPHP-RC patients exhibit various combinations of extrarenal manifestations, including liver fibrosis, retinal degeneration, coloboma, cerebellar vermis aplasia, and polydactyly among others (Hildebrandt, Attanasio and Otto, 2009). Patients normally progress to KF before the age of 30 years. The poor genotype-phenotype correlation makes the diagnosis of NPHP-RC difficult. In some cases, NPHP-RC patients can show ARPKD-like enlarged kidneys or even Potter-like characteristics. The first genetic cause identified was the gene *NPHP1* (Hildebrandt *et al.*, 1997; Saunier *et al.*, 1997). The homozygous deletions in the *NPHP1* gene are the most frequent cause of NPHP-RC, accounting for 20-25% of all cases (Halbritter *et al.*, 2013). Currently, more than 20 genes are known to cause NPHP-RC. Each of the subsequently identified monogenic genes accounts only for a small fraction of affected individuals (Halbritter *et al.*, 2013).

Disease-causing variants in the *HNF1B* gene can also cause renal cysts. Heterozygous disease-causing variants of this gene were first described in patients with maturity-onset diabetes of the young (MODY) (Horikawa *et al.*, 1997). Subsequently, various kidney phenotypes have been associated with disease-causing variants in this gene, encompassing CAKUT and tubular transport disorders (Madariaga *et al.*, 2019). The *HNF1B* gene has also been linked to ciliopathies through its direct effect on gene regulation. Indeed, *PKD2* and *PKHD1* are under the transcriptional control of *HNF1B* (Gresh *et al.*, 2004). Thus, some clinical findings in patients with *HNF1B* disease-causing variants can mimic those found in cystic kidney diseases (such as ADPKD, ARPKD, and NPHP-RC). Variable penetrance with non-specific clinical manifestations and a high prevalence of *de novo* cases complicates more the diagnosis.

Renal cysts can also be clinical manifestations in multisystemic diseases such as tuberous sclerosis complex (TSC), autosomal dominant polycystic liver disease, oro-facial-digital syndrome, and renal coloboma syndrome among others (Cramer and Guay-Woodford, 2015).

Studies based on the parallel sequencing of more than 100 cystic genes detected a monogenic cause in 50–70% of young patients with two or more hyperechoic cysts and/or kidneys by ultrasound (Halbritter *et al.*, 2012, 2013; Gee *et al.*, 2014; Braun *et al.*, 2015; Bullich *et al.*,

2018). In several NGS studies, *PKD1* is not analyzed due to the high complexity of its sequencing (Mallett *et al.*, 2017; Lata *et al.*, 2018; Connaughton *et al.*, 2019).

2.1.3. Glomerulopathies

Monogenic glomerulopathies are a group of diseases that manifest with proteinuria or microhematuria or both caused by structural defects in the glomerular basement membrane or the podocytes. Currently, more than 60 genes responsible for glomerulopathies have been described. The distribution of the different inherited glomerulopathies according to the age at disease onset is shown in Figure 5.
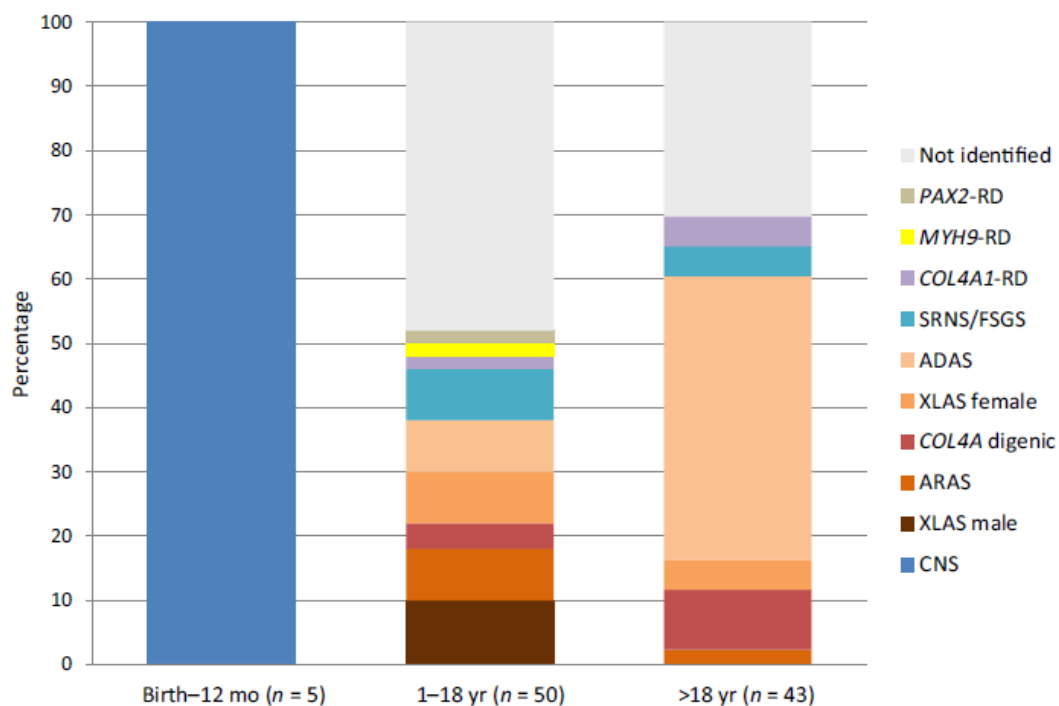
Figure 5. Distribution of inherited glomerulopathies according to the age at disease onset. ADAS, autosomal dominant Alport syndrome; ARAS, autosomal recessive Alport syndrome; CNS, congenital nephrotic syndrome; FSGS, focal segmental glomerulosclerosis; RD, related disease; SRNS; steroid-resistant nephrotic syndrome; XLAS, X-linked Alport syndrome. (Bullich *et al.*, 2018).

Alport syndrome (AS) is the second commonest cause of IKD after ADPKD. It accounts for at least 1–2% of all cases of patients on KRT (Gretz *et al.*, 1987; Mallett *et al.*, 2014).The typical clinical features of AS are persistent microscopic hematuria, non-nephrotic range proteinuria, KF, and often a family history of hematuria or KF. Hearing loss is common, and ocular abnormalities may be present (Gubler *et al.*, 1981). It can be caused by disease-causing variants in the *COL4A5* (X-linked AS), *COL4A3*, or *COL4A4* genes (AD and AR AS) (Kruegel, Rubel and Gross, 2013). The existence of three different patterns of inheritance in part explains the

wide spectrum of disease, ranging from isolated microhematuria to KF. Male patients with X-linked AS (XLAS) and patients with autosomal recessive AS (ARAS) typically have more severe outcomes at earlier age, while women with XLAS show a milder phenotype, as it is the case for most X linked disorders, and also variable due to the X inactivation phenomenon (Jais *et al.*, 2003). Patients with heterozygous disease-causing variants in *COL4A3* or *COL4A4* genes display a high variability of manifestations, ranging from asymptomatic to presentation with hematuria alone or with proteinuria and subsequent KF on top of hematuria. There is a lot of debate over how to designate the disease caused by heterozygous variants in the *COL4A3* or *COL4A4* genes. Meanwhile the term "thin basement membrane nephropathy" is not longer recommended since it is an histopathological finding rather than a distinct disease entity, the term autosomal dominant AS (ADAS) is becoming more accepted among the nephrologists (Kashtan *et al.*, 2018; Torra and Furlano, 2019; Furlano *et al.*, 2021; Kashtan, 2021). Disease-causing variants in other genes can produce similar clinical features to AS, such as *MYH9* and *COL4A1* (Plaisier and Ronco, 2016; Savoia and Pecci, 2021).

Nephrotic syndrome (NS) encomprises a heterozygous group of disorders characterized by nephrotic range proteinuria, hypoalbuminemia, +/- presence of edema (Noone, Iijima and Parekh, 2018), that affects about one to three per 100,000 children aged below 16 years (McKinney *et al.*, 2001; Dossier *et al.*, 2016). Approximately 85% of paediatric NS cases respond to glucocorticoids treatment, with the remaining 15% being sterioid-resistant (Lombel, Gipson and Hodson, 2013).

Steroid-resistant NS (SRNS) of genetic origin is mostly resistant to immunosuppressive agents, almost invariably progresses to KF, and does not relapse after renal transplantation (Machuca, Benoit and Antignac, 2009). SRNS has a high genetic heterogeneity, with over 50 causative genes described to date, being *NPHS2, NPHS1*, and *WT1* the most common ones (Santín *et al.*, 2011; Sadowski *et al.*, 2015). Monogenic SRNS can be inherited in an AR, AD or mitochondrial manner, and can occur as isolated renal disease or as part of a syndrome.

Disease-causing variants screening in large cohorts of patients with SRNS revealed that the distribution of the causative genes depends on the age at onset, the familial/sporadic status, and the mode of inheritance. In patients with SRNS the genetic diagnostic rate is inversely correlated with age of onset, ranging from almost 100% of patients with congenital NS to around 30% of families manifesting before 25 years of age (Figure 6), and much lower in sporadic and adult cases (Santín *et al.*, 2011; Sadowski *et al.*, 2015). However, monogenic contribution in adult-onset SRNS/FSGS without positive family history may be underestimated

and could be higher than previously considered as Gribouval *et al.* reported a diagnostic rate of >20% (Gribouval *et al.*, 2018). Most of the genes involved in paediatric SRNS follow an AR inheritance, including disease-causing variants in *NPHS1*, *NPHS2*, and *PLCE1* among others. The exception is *WT1*-related SRNS which follows an AD pattern. The AD forms of SRNS (*INF2*, *TRPC6* among others) are generally characterized by a milder disease course with typically adolescent (from 13 to 18 years) or adult (>18 years) onset, variable degrees of proteinuria, FSGS on renal biopsy, and slow progression to KF (Conlon *et al.*, 1995; Rana *et al.*, 2003; Santín *et al.*, 2011).

FSGS represents the most common renal histology in paediatric patients with SRNS (Trautmann *et al.*, 2015). Adolescent and adult-onset familial FSGS has been found to be mostly caused by disease-causing variants in *COL4A3, COL4A4, NPHS2* (p.R229Q variant *in trans* with specific pathogenic variants), *INF2*, *PAX2*, and *TRPC6* (Santín *et al.*, 2011; Barua *et al.*, 2014; Malone *et al.*, 2014; Gast *et al.*, 2016). However, a poor correlation between the histological findings and the underlying genotype has been reported (Bierzynska *et al.*, 2017).
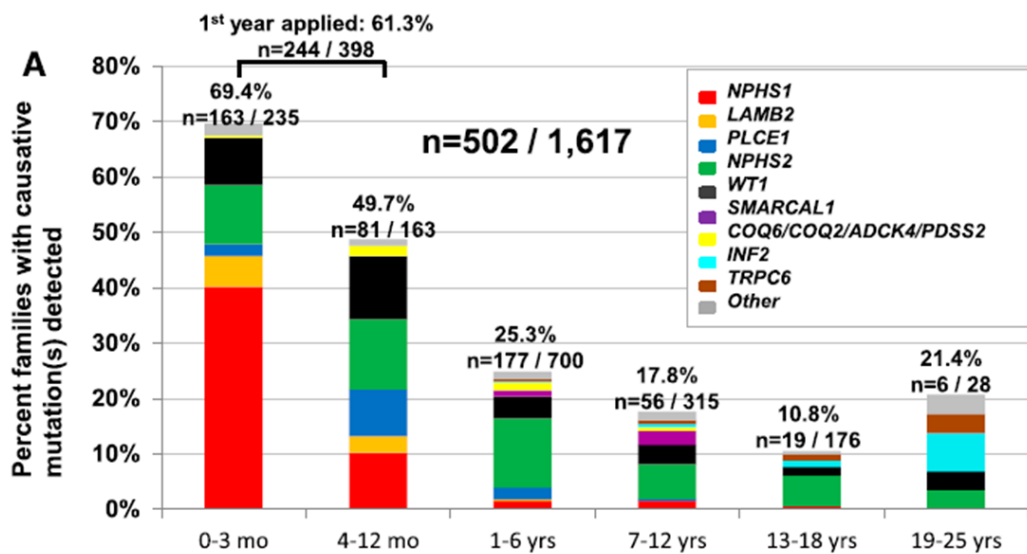
Figure 6. Percentage of patients with causative variant detected in one of the 21-SRNS genes analyzed in relation to age of onset of proteinuria. Mo, months; yrs, years (Sadowski *et al.*, 2015).

2.1.4. Tubulopathies

Tubulopathies are a group of disorders caused by dysfunction of proteins involved directly or indirectly in the epithelial transport along the renal tubules. Many of these proteins are solute carrier membrane transport proteins with an important role in body homeostasis adjusting the reabsorption and secretion of water and solutes (Ashton *et al.*, 2018). To date more than 60

genes have been linked to tubulopathies. Although they are often diagnosed in childhood, they can also first manifest in adulthood.
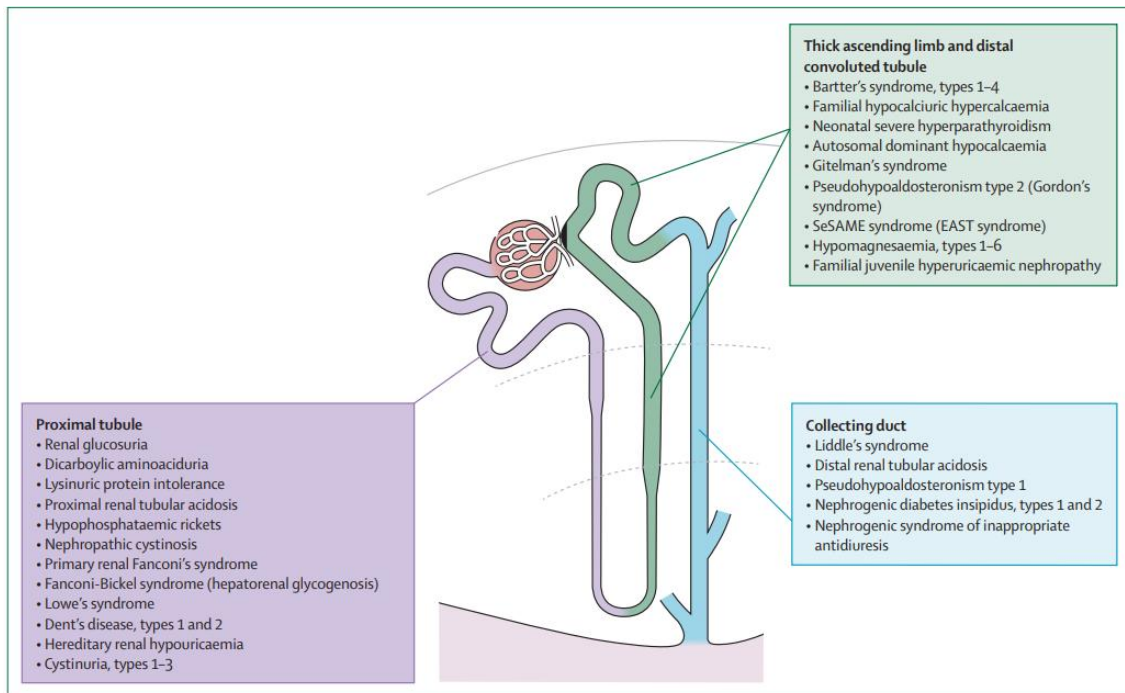
Figure 7. Inherited tubulopathies linked to nephron segments. Adapted from (Devuyst *et al.*, 2014)**.**

Based on anatomic and functional characteristics, the tubules are typically divided into four main segments: proximal tubule, Henle's loop, distal convoluted tubule, and collecting duct. Depending on the tubular function of the nephron that is affected, each specific disorder has its own age of onset, clinical and analytical manifestations, severity, and prognosis (Figure 7). Some of the conditions affecting the proximal tubule are cystinuria (caused by disease-causing variants in the *SLC3A1*, *SLC7A9* and *SLC7A13* genes), Dent's disease (caused by disease-causing variants in the *CLCN5* and *OCRL1* genes), X-linked hypophosphatemic rickets (caused by disease-causing variants in the *PHEX* gene), and AR proximal renal tubular acidosis (caused by disease-causing variants in the *SLC4A4* gene). The main tubulupathies affecting the Henle's loop are Bartter syndrome (caused by disease-causing variants in the *BSND, CASR, CLCNKA, CLCNKA, KCNJ1, SLC12A1* genes) and hypomagnesemia with hypercalciuria and nephrocalcinosis (caused by disease-causing variants in the *CLDN16* and *CLDN19* genes). Diseases affecting the distal and collecting tubules are Gitelman syndrome (caused by disease-causing variants in the *SLC12A3* gene), distal renal tubular acidosis (caused by *ATP6V1B1*, *ATP6V0A4* and *SLC4A1* genes), Liddle syndrome (caused by disease-causing variants in the *SCNN1B* and *SCNN1G* genes), nephrogenic diabetes insipidus (caused by disease-causing variants in the *AVPR2* and *AQP2* genes), among others. Cystinosis (caused by disease-causing variants in the *CTNS* gene) affects all tubular segments. A monogenic cause can be detected in

about two thirds of patients with paediatric onset tubulopathy, but this decrease to one third in those with adult onset (Ashton *et al.*, 2018; Hureaux *et al.*, 2019).

2.1.5. ADTKD

Autosomal dominant tubulointerstitial kidney disease (ADTKD) is a rare, but underdiagnosed, cause of kidney diseases characterized by interstitial fibrosis and secondary tubular damage in the absence of glomerular lesions, AD inheritance, and slowly progressive CKD. Affected individuals develop CKD achieving KF usually between the third and sixth decade of life (Bleyer and Kmoch, 2014; Ayasreh *et al.*, 2018).

In 2014, KDIGO (Kidney Disease: Improving Global Outcomes) proposed the adoption of a new terminology for this group of diseases in which the term "ADTKD" is appended by a gene-based subclassification and also suggested diagnostic criteria (Eckardt *et al.*, 2015). These diseases are clinically similar but are caused by disease-causing variants in at least 5 different genes: *MUC1*, *UMOD, HNF1B, REN*, and *SEC61A1* (Table 1). There is no evidence establishing the prevalence of the different types of ADTKD, but the most frequently causative genes are *UMOD* and *MUC1*. A diagnostic rate of 45% has been established in a Spanish cohort of 56 families with suspected ADTKD. Of those, *MUC1* was the most prevalent cause (64%; 16/25), followed by *UMOD* (36%; 9/25), while no disease-causing variants were identified in *REN* nor *HNF1B* (Ayasreh *et al.*, 2018). The predominant pathogenic variant in the *MUC1* gene is a cytosine duplication causing a frameshift located in a tandem repeat region [*MUC1* (NM_001204286.1): c.428dupC p.(Ala144Serfs*86)].

Table 1. Specific features of each subtype of ADTKD

ADTKD subtype	Specific clinical features	Laboratory features	Pathological features
ADTKD-*UMOD*	• Gout as early as the teenage years • Enuresis and urinary concentrating defects	• Hyperuricaemia due to low FEurate • Low urinary levels of uromodulin	Intracellular deposits of uromodulin in cells lining the TAL
ADTKD-*MUC1*	Gout*	Hyperuricaemia*	Intracellular deposits of *MUC1fs* in cells lining the TAL and in extra- renal tissues
ADTKD-*HNF1B*	• Frequent presentation in childhood • CAKUT • Genital abnormalities	• Hypomagnesaemia, hyperuricaemia and hypokalaemia • Abnormal liver	NA

	in women • Syndromic features such as autism spectrum disorder (in those with 17q12 deletion syndrome)	function tests • MODY	
ADTKD-*REN*	• Childhood anaemia • Mild hypotension • Propensity to develop acute kidney injury • Gout in adolescence	• Childhood anaemia • Mild hyperkalaemia • Hyperuricaemia due to low FEurate • Low or low–normal plasma renin levels	Reduced renin staining in the juxtaglomerular apparatus
ADTKD-*SEC61A1*	• Intrauterine and postnatal growth retardation • Bifid uvula • Cleft palate and velopharyngeal insufficiency • Pre- axial polydactyly • Abscess formation	• Congenital anaemia • Leukopenia and neutropenia	NA

Abbreviation: ADTKD, autosomal dominant tubulointerstitial kidney disease; CAKUT, congenital anomalies of the kidney and the urinary tract; FEurate, fractional excretion of urate; MODY, maturity-onset diabetes of the young; NA, not available; TAL, thick ascending limb (of the loop of Henle). *Less frequent than in ADTKD-UMOD (Devuyst *et al.*, 2019).

2.1.6. CKD of unknown aetiology

Achieving a precise clinical diagnosis in patients with CKD is not always straightforward due to the high phenotypic overlap in some CKD causes or atypical clinical presentations. In many cases after the traditional diagnostic workup (including history and physical examination, biochemical testing, renal imaging, or renal biopsy) the aetiology of the CKD remains elusive. CKD of unknown aetiology represents approximately 5–25% of patients with KF (Saran *et al.*, 2020; Torra *et al.*, 2021). Recent studies indicate that genetic testing can establish the genetic cause in approximately 10–40% of cases with CKD of unknown aetiology (Groopman *et al.*, 2018; Lata *et al.*, 2018; Connaughton *et al.*, 2019; Mann *et al.*, 2019; Ottlewski *et al.*, 2019).

2.1.7. Genetic diagnostic yield

Several studies have screened for a monogenic cause in cohorts of patients with CKD, achieving diagnostic rates ranging from 6–30% for adults (Groopman *et al.*, 2018; Ottlewski *et al.*, 2019; Yao *et al.*, 2019) and 30% for paediatric cohorts (Mann *et al.*, 2019). Higher diagnostic yields of 37% to 59% have been reported in cohorts of patients with suspected IKD

(Mallett *et al.*, 2017; Mori *et al.*, 2017; Lata *et al.*, 2018; Mansilla *et al.*, 2019; Jayasinghe *et al.*, 2021). The differences among the reported diagnostic yields in different studies are mainly due to the selection criteria of the study cohort, including age at onset, clinical diagnostic group of kidney disease, number of familial/sporadic cases, presence or not of extrarenal manifestations. The diagnostic yield also depends on the technology used, the selection of genes analyzed and the coverage and depth of coverage of the targeted regions obtained in the massive parallel sequencing.

2.2. EXPANDING RENAL DISEASE PHENOTYPES

Genetic overlap is common among different kidney disease. Genomic studies in nephrology have broadened the understanding of the phenotypic spectrum associated with some genes that can cause kidney diseases belonging to different clinical diagnostic groups and within the same clinical disease group. For instance, disease-causing variants in *PAX2* were first associated with CAKUT as a part of a syndrome known as renal coloboma (Ecoles and Schimmenti, 1999). They were also identified in patients with isolated CAKUT (Nishimoto *et al.*, 2001; Weber *et al.*, 2006). Later, *PAX2* have been associated with autosomal dominant FSGS (Barua *et al.*, 2014). Similarly, disease-causing variants in the *HNF1B* gene can cause a spectrum of related diseases, but were first described in patients with MODY and renal cysts (Horikawa *et al.*, 1997). Several anomalies were also associated to this gene including CAKUT (mainly bilateral cystic dysplasia) (Heidet *et al.*, 2010), and also as a cause of ADTKD. Disease-causing variants in *HNF1B* gene can also mimic the phenotype of other cystic ciliopathies as ADPKD and ARPKD.

Phenotypic heterogeneity has also been observed in other genes that can be responsible for different kidney disease such as *COL4A3/4/5* genes causative of AS and have been reported in families with a diagnosis of AD FSGS (Xie *et al.*, 2014; Gast *et al.*, 2016), and *TTC21B* causative of NPHP-RC and FSGS (Davis *et al.*, 2011; Cong *et al.*, 2014; Bullich *et al.*, 2017). The list of genes with a broad phenotype spectrum is continuously growing (for instance, *CUBN* (Bedin *et al.*, 2020), and *DGKE* (Lemaire *et al.*, 2013; Ozaltin *et al.*, 2013) as more patients undergo genetic testing.

This clinical variability has led some nephropathies to be named in relation to their specific genetic cause, such as "*HNF1B*-associated kidney disease" (Clissold *et al.*, 2015), "nephropathy-*PAX2* related" (Bullich *et al.*, 2018) or "ADTKD-*UMOD*" (Eckardt *et al.*, 2015). In fact, Clinical Genome Resource (ClinGen), the National Institutes of Health-funded resource dedicated to

building a central resource that defines the clinical relevance of genes and variants for use in precision medicine and research, and KDIGO, a global organization developing and implementing evidence based clinical practice guidelines in kidney disease, encourage the development of terminologies including the gene name in addition to the clinical name in order to unified diseases terminologies. However, to include or not the gene name remains a matter of debate.

2.3. POTENTIAL CAUSES OF PHENOTYPIC VARIABILITY

Currently, it is unknown why patients with the same monogenic kidney disease often have very different clinical presentations. Some of the factors that may contribute to this high phenotypic variability are the following (Figure 8) (Ars and Torra, 2017):
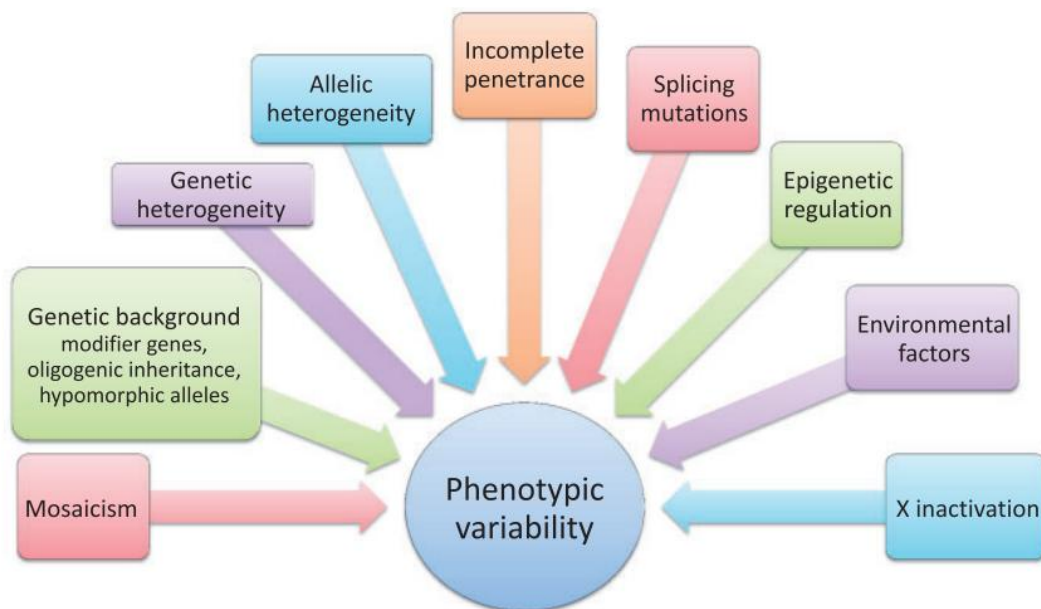
Figure 8. Possible explanations for phenotypic variability in IKD (Ars and Torra, 2017)**.**

a) Genetic and allelic heterogeneity

Genetic heterogeneity occurs in most monogenic kidney diseases, which means that different genes can be causative of the same disease and depending on the causative gene the phenotype may differ. A clear example is ADPKD, where patients with *PKD1* disease-causing variants reach KF about 20 years before than those with *PKD2* disease-causing variants (Hateboer *et al.*, 1999). Other examples are ADTKD, which can be caused by disease-causing variants in *MUC1*, *UMOD, HNF1B, REN*, and *SEC61A1* with similar phenotypes (Eckardt *et al.*,

2015), and recessive disease-causing variants in *COL4A3* and *COL4A4* that cause similar phenotype as *COL4A5* in AS males.

Allelic heterogeneity refers to the different variants of the same gene (alleles), which can give rise to different phenotypes. In general, genotype–phenotype correlations are weaker for autosomal dominant monogenic disorders than for autosomal recessive diseases. For instance, carrying two truncating variants in the *PKHD1* gene is associated with a more severe phenotype of ARPKD, which can be lethal, than carrying at least one missense disease-causing variant (Gunay-Aygun *et al.*, 2010).

b) Penetrance and expressivity

Penetrance is defined as the proportion of individuals with a certain disease-causing variant who display the disease phenotype. If the genotype is always expressed, penetrance is complete. Meanwhile, incomplete penetrance refers to the observation that some individuals with the genotype do not develop the disease phenotype. In general, for most recessive diseases penetrance is complete while for dominant diseases penetrance is often incomplete (at least up to a certain age). An example of incomplete penetrance is patients with heterozygous disease-causing variants in *COL4A3* or *COL4A4* genes, which display a wide spectrum of manifestations, ranging from asymptomatic to presentation with hematuria alone or with proteinuria and subsequent KF (Furlano *et al.*, 2021).

Variable expressivity refers to different degrees of severity and/or organ involvement in different affected individuals that carry an identical disease-causing variant. This phenotypic variability can be interfamilial (among unrelated individuals) and intrafamilial (among individuals of the same family). For instance, patients with heterozygous disease-causing variants in *COL4A3* or *COL4A4* show a huge inter- and intrafamilial variability. It should be noted that many diseases have variable expressiveness with age, so that a very mild phenotype in childhood does not exclude a moderate or severe development of the disease.

Incomplete penetrance and variable expressivity may be related on the existence of modifier genes and the influence of environmental factors, which modulate the effect of the main disease-causing gene.

c) Oligogenic inheritance and modifier genes

Oligogenic inheritance occurs when the disease is caused by pathogenic variants in more than one gene. It implies that these few genes exert an effect of comparable magnitude on the phenotype. Such inheritance has been suggested but not clearly demonstrated yet in some IKD such as Bardet–Biedl syndrome (Katsanis, 2004), NPHP-RC (Hoefele *et al.*, 2007) and AS (Mencarelli *et al.*, 2015).

The term "modifier variant" is used for a sequence variant that is supposed not to be a causative variant but that contributes to the disease phenotype. Modifier variants can be located either in the causative gene, in addition to the disease-causing variant, or in other genes involved in common pathways. Modifier genes probably play a relevant role in explaining part of the variable expressivity among patients carrying the same disease-causing variant, especially in adult-onset diseases such in AS, ADPKD and ADTKD. Studies of families with ADPKD with members presenting with an early and severe disease have been found to carry an incompletely penetrant (hypomorphic) *PKD1* allele *in trans* with the familial *PKD1* disease-causing variant. Hypomorphic variants by themselves give no phenotype or only a very mild one (e.g. few cysts), but together with another hypomorphic variant or pathogenic variant *in trans* worsen the severity of the disease (Rossetti *et al.*, 2009; Vujic *et al.*, 2010).

d) Mosaicism

Mosaicism is a presence of two or more population of cells with different genotypes derived from a single zygote in a single individual. Depending on the expression of the mutated allele, in terms of both percentage and organ-specific expression, different phenotypes arise. There are 3 types of mosaicism: somatic, germinal, and gonosomal, depending on whether it affects somatic cells, germ cells, or both. Mosaicism can explain milder phenotype of disease in a sporadic case. It also has to be taken into account during reproductive genetic counselling of the healthy parents of a *de novo* case of an AD IKD. The parents could be counselled that the recurrence risk for a second affected child is extremely low, as the first child is supposed to carry a *de novo* pathogenic variant. However, if one of the parents present germinal mosaicism (coexistence of mutated and non-mutated eggs or sperm), the couple may conceive another affected child. Mosaic pathogenic variants can be difficult to detect by Sanger sequencing. The high-throughput nature of NGS technology allows for very high depth of coverage, with detection of a low percentage of the disease-causing variant in respect to the percentage of the wild-type allele. TSC is an example of an AD disease where *de novo* pathogenic variants are

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common, accounting for 60-70% of cases (Sampson *et al.*, 1989). These new disease-causing variants usually happen in germ cells but can occur postzygotically, resulting in mosaicism.

e) X inactivation

This is a dosage compensation mechanism in which one of the copies of the X chromosome present in a female is inactivated. The inactive X chromosome is silenced and is transcriptionally inactive. The choice of which of the two X-X chromosomes is inactivated is a random process in humans that occurs in each cell during early female embryonic development. All the descendant cells of each of these embryonic cells will maintain the same pattern of inactivation of the X chromosome. In certain cases the inactivation is biased towards the wild-type or the mutated allele. If there is a high percentage (>90%) of cells with the wild-type allele inactivated, the disease is much more severe than would be expected for a female (Vetrie *et al.*, 1992; Migeon, 2008). On the other hand, if there is a high percentage of cells with the mutated X chromosome inactivated, the woman will be practically asymptomatic. This phenomenon is organ specific; thus the findings in one cell type, or organ, cannot be extrapolated to other organs. Some examples of X-linked IKD where X-inactivation may explain the phenotypic variability are Fabry disease (Echevarria *et al.*, 2016), X-linked AS (Jais *et al.*, 2003), and Dent's disease (Mansour-hendili *et al.*, 2015).

f) Splicing variants

Splicing is the process by which introns (the noncoding regions of the gene) are excised out of the primary messenger RNA transcript, and the exons (the coding regions of the gene) are joined together to generate mature messenger RNA that serves as the template for synthesis of a specific protein.

Clinical variability among patients carrying the same splicing variant has been related to variable levels of aberrantly spliced transcripts (Nissim-Rafini and Karem, 2005). This phenomenon may occur especially in splicing variants that do not affect the intronic canonical splice sites (GT/AG). This type of splicing variants generates a variable proportion of wild-type transcript, in addition to the aberrantly spliced transcript. The molecular basis of the phenotypic differences between and within families carrying the same non-canonical splicing variant could be explained by the variation in the levels of aberrant transcript. The higher the proportion of the aberrantly spliced transcript, the more severe is the disease phenotype expected to be. Splicing variants can also cause the truncation of the protein. In males affected

by X-linked AS, it has been reported that the phenotype is more severe whether the disease-causing variant alter the canonical splicing sequences (Jais *et al.*, 2003) or if the splicing variants generate a truncated protein (Horinouchi *et al.*, 2018).

g) Epigenetic regulation

Epigenetics refers to heritable changes that are not caused by alterations in the nucleotide sequence itself. The most common studied mechanisms of epigenetic modification include DNA methylation at CpG dinucleotides, histone acetylation, histone phosphorylation, and histone methylation. These modifications modulate the structure of the chromatin and change its accessibility to transcription factors, allowing certain regions to become accessible (or inaccessible) to transcription factors and consequently for genes to be expressed or silenced. Any type of cell has specialized epigenetic patterns, and epigenetic patterns change with ageing, environmental factors, diseases, among others. Epigenetics is an emerging field of science, so far little studied in IKD. However, numerous studies support that epigenetic and gestational environmental risk factors can influence kidney development and/or fibrosis and might also increase susceptibility to CAKUT.

h) Environmental factors

Phenotype is impacted by environmental factors experienced during embryonic development and throughout life. These factors are many and varied, and include diet, climate, drugs, illness, and stress, among others. Although little is known, it is universally accepted, for example, that an inadequate diet may cause obesity, diabetes and hypertension, and that these conditions will worsen the IKD phenotype. For instance, the complex aetiology of CAKUT implies that both genetic and environmental factors contribute to the natural history of disease; we thus also still need to understand the effects of several environmental factors before and during pregnancy in the mechanism of disease (Nicolaou *et al.*, 2015).

2.4. GENOMIC DISORDERS

Several studies have noted enrichment of genomic disorders among paediatric patients with CAKUT and other kidney phenotypes, supporting a broader contribution of genomic imbalances to CKD (Verbitsky *et al.*, 2015, 2019).

Of the genomic disorders identified in individuals with CKD, CNV at the 17q12, 16p11.2 and 22q11.2 loci are among the most common, estimated to collectively occur in 2.9% of patients

with CAKUT (Sanna-Cherchi *et al.*, 2018). As these loci contain multiple genes that have pleiotropic effects, each of these disorders has diverse multiorgan manifestations but share the feature of being associated with highly variable neurodevelopmental, cardiac and renal anomalies.

17q12 deletion syndrome: was first detected among individuals with renal cysts and/or CAKUT and MODY (Bellanné-Chantelot *et al.*, 2005). It was subsequently found to be enriched among patients with neurological disorders, such as autism, schizophrenia and epilepsy; other manifestations can include dysmorphic facial features, liver disease, and cardiac, musculoskeletal and gastrointestinal anomalies (Moreno-De-Luca *et al.*, 2010; Nagamani *et al.*, 2010; Mitchel *et al.*, 2020). This deletion is highly penetrant, but expressivity is variable and significant intrafamilial variability has been observed. The most common features identified in more than 50% of patients carrying this deletion are kidney structural or functional defects, being cystic dysplasia the most common, neurodevelopmental/neuropsychiatric disorders, mild dysmorphic features, and hyperparathyroidism. The classic 17q12 deletion syndrome interval contains 15 genes, including *HNF1B*. It also contains the genes *LHX1* and *ACACA*, which are thought to contribute to the neurological anomalies observed in most of the patients with this deletion.

The spectrum of severity and range in age of detection of *HNF1B*-associated kidney disease are broad, including prenatal severe KF, slow progression to KF in adulthood, and normal kidney function never requiring KRT (Madariaga *et al.*, 2013; Clissold *et al.*, 2015; Verhave *et al.*, 2016). Recent evidences suggested that intragenic *HNF1B* pathogenic variants may be associated with worse kidney function and higher risk of progression to KF compared to 17q12 deletions (Dubois-Laforgue *et al.*, 2017; Clissold *et al.*, 2018). The reason for this finding is unknown, but the authors speculate a possible dominant-negative effect of certain *HNF1B* variants resulting in a more severe phenotype, or a protective effect conferred by the loss of one or more genes in the 17q12 recurrent deletion region.

16p11.2 microdeletion syndrome: was first recognized as a recurrent genomic disorder among individuals with autism spectrum disorder, and were subsequently found to have extraneurological phenotypes including obesity, congenital heart defects, vertebral anomalies, macrocephaly and hearing impairment. This microdeletion has been also detected in multiple patients with CAKUT in microarray studies of paediatric CKD cohorts (Verbitsky *et al.*, 2015; Sanna-Cherchi *et al.*, 2018). It has been demonstrated that CAKUT is a major feature of the 16p11.2 microdeletion syndrome and the *TBX6* gene has been identified as a key driver of the

renal phenotypes observed (Verbitsky *et al.*, 2019). This recurrent microdeletion involves the loss of one chromosomal segment harboring 25 genes.

22q11.2 deletion syndrome: is the most common chromosomal microdeletion disorder, estimated to occurs as a *de novo* 1.5–3 Mb deletion in most individuals (Lindsay *et al.*, 1995; Morrow *et al.*, 2018), although approximately 5% are inherited (McDonald-McGinn *et al.*, 2001). It is associated with DiGeorge syndrome, velocardiofacial syndrome and conotruncal anomaly face syndrome. The manifestations of this disorder include congenital heart defects, immunological alterations, hypocalcaemia, craniofacial and musculoskeletal anomalies, malignancies, neuropsychiatric disease; and renal alterations in about 30% of cases (McDonald-McGinn, 2020). The most frequent renal manifestations are hypodysplasia and renal agenesis, although it has been detected in patients with horseshoe kidneys, multicystic dysplasia, and hydronephrosis. Individuals with this deletion can present with a wide range of features that are highly variable; while some patients are mildly affected, others have severe medical, cognitive, and/or psychiatric challenges.

The 22q11.2 locus is a 3-Mb region that includes four sets of low copy number repeats (LCRs), designated LCR22A, LCR22B, LCR22C and LCR22D. Heterozygous 2.5-Mb deletions spanning regions A–D (LCR22A–D) are detected in over 90% of affected individuals. There are 45 known protein coding genes, seven miRNA and 10 noncoding genes that map to the 3 Mb deletion. Prior studies identified *TBX1*, located in the LCR22A–B interval, as a candidate gene for DiGeorge syndrome. However, the fact that the syndrome can result from smaller 22q11.2 deletions that do not include *TBX1* supports a role for other genes in the pathogenesis of the DiGeorge phenotype, including its renal manifestations. Besides *TBX1*, *CRK* is another candidate gene. *CRKL* maps to the LCR22C-D region and it encodes a cytoplasmic adaptor protein involved in growth factor signaling. Recently, it was found that hemizygosity of *CRKL* contributes to genitourinary tract development in humans and animal models, including the kidney (Haller *et al.*, 2017; Lopez-Rivera *et al.*, 2017). Besides coding genes, non-coding genes, such as miRNAs might also contribute to the etiology of 22q11.2 deletion syndrome.

2.5. CKD AS A COMPLEX DISEASE

Monogenic forms of disease are usually caused by rare or even private variants in a single gene that have a large effect size. Polygenic subtypes of kidney disease usually reflect the collective contribution of multiple common variants, each of which has a small effect on disease risk. However, in some cases, relatively common variants have been shown to have a large impact

on the disease, such as *APOL1* risk variants and various forms of non-diabetic CKD (Genovese *et al.*, 2010; Tzur *et al.*, 2010).

GWAS involve testing genetic variants across the genomes of many individuals to identify genotype-phenotype associations. Since the early 2000s, GWAS have revolutionized the field of complex disease genetics providing numerous compelling associations for human complex traits and diseases. GWAS in nephrology have so far focused in the study of specific CKD aetiologies, such as IgA nephropathy (IgAN) or membranous nephropathy (MN), and the study of CKD-defining traits, eGFR and the Urinary albumin/creatinine ratio (UAlb/Cr). Common risk variants of large effect can be identified in GWAS of <100 individuals in MN, whereas GFR-defined CKD is heterogeneous and population-based studies require >20,000 individuals to detect associated loci of small effect. For instance, GWAS have reported highly significant association of an *HLA-DQA1* allele on chromosome 6p21 and a *PLA2R1* allele on chromosome 2q24 with idiopathic MN in patients of white ancestry. Interestingly, carrying the risk alleles of both genes had an additive effect, thus, the odds ratio for developing idiopathic MN is close to 80 for a person who is homozygous for both risk alleles, compared to individuals homozygous for the protective alleles (Stanescu *et al.*, 2011). As another example, GWAS studies have identified several variants in the *CUBN* gene associated with increased risk of albuminuria (Böger *et al.*, 2011; Teumer *et al.*, 2016; Haas *et al.*, 2018; Ahluwalia *et al.*, 2019; Zanetti *et al.*, 2019).

3. GENETIC COUNSELING

Genetic counseling is a communication process, which aims to assist affected or at-risk individuals, couples and families in understanding the natural history, disease risks, and mode of transmission of a genetic disorder (Resta *et al.*, 2006). This process involves obtaining a detailed medical and family history, performing a risk assessment for the patient, and providing education, psychosocial counseling, and support. The implications of a genetic diagnosis on the patient's prognosis, medical management, familial risk, and risk of recurrence following kidney transplant are also discussed. Appropriate pre-test counseling on the opportunities, limitations and possible results of the genetic testing allows patients or parents of an affected child to make an informed decision on whether or not to undergo genetic testing and to understand the potential outcomes. Once the genetic test report is available, the test results are communicated to the patient and the genetic counselor should be sure that the patient understands the implications of the results.

It is important to note that in some cases identified genetic variants may not rise to the level of pathogenicity required to establish causality. Therefore, additional studies including segregation analysis within an affected family or data sharing can provide further evidence to support pathogenicity of the identified variant. The chance of identifying these variants should be emphasized, and also the potential of becoming meaningful overtime. The counseling should also include the chance of incidental findings. In addition, a negative result neither confirms nor discards the suspected clinical diagnosis and it has to be interpreted with caution taking into account the different limitations of the technique used. For instance, since gene-panels and WES typically focus on coding exons and their associated splice sites, a negative result may also arise because the causal variant is a deep intronic variant not captured in the sequencing strategy.

3.1. INFORMED CONSENT

Informed consent is a required component of all clinical and research genetic testing. It is a legal document that must be signed by the patient. It recognizes the patient's right to information and the 'right not to know', outlining the duty of confidentially and the right to the protection of the data; it warns against the possibility of incidental findings and allows the patient to make a decision on receiving or not its communication; it cautions that the information obtained may have implications for family members and when it is appropriate to convey information to them; and it states the compromise to provide genetic counseling (Pàmpols *et al.*, 2016). Whenever possible, children and adolescents should assent for genetic testing, but in case of young children with lack decision-making capacity, the decisions about testing must be conducted through surrogates, usually their parents, and must be done with the child's best interest. Normally, testing for late-onset disorders in minors are postponed until the individual can provide full informed consent.

3.2. INDICATIONS FOR GENETIC TESTING AND CLINICAL APPLICATIONS

The identification of the genetic cause of CKD has several clinical implications as it provides an unequivocal molecular diagnosis for the patients and their family members. For that reason genetic testing is becoming an important contributor to clinical decision making. The main clinical applications of genetic testing are confirmation of a suspected clinical diagnosis, identification of the molecular cause of CKD of unknown aetiology, carrier screening, presymptomatic, preimplantation and prenatal diagnosis.

Diagnostic testing is used to diagnose a specific genetic or chromosomal condition. In many cases, genetic testing is used to confirm a diagnosis when a particular condition is suspected. It can also play an essential role when the clinical phenotype does not suggest a clear diagnosis. Diagnostic testing can be performed at any time during a person's life. The results of a diagnostic test can influence a person's choices about health care and the management of the disease confirming clinical diagnosis, establishing inheritance patterns, differentiating heterogeneous disorders, determining appropriate treatment, guiding decisions about family planning, determining the cause of unexplained familial renal disorders, identifying risk factor for recurrence in kidney transplantation, evaluating family members' suitability for kidney donation, and prompting evaluation for extrarenal features.

Carrier screening is used to identify asymptomatic individuals who carry variants associated with X-linked and AR genetic diseases. This kind of testing is used most often by couples who are considering becoming pregnant to determine the risks of their child inheriting one of these genetic disorders.

Presymptomatic diagnosis is the performance of a genetic test on an asymptomatic individual at risk of a condition to determine whether the person has inherited the disease-causing variant(s). If the disorder is known in the family, presymptomatic or predictive testing is offered to "at-risk" symptomless persons. Reassurance or future life planning, selecting a living related kidney donor, screening or preventative treatment, and the relief of uncertainty are the major reasons for such testing.

Preimplantation genetic diagnosis is performed on embryos resulting from in vitro fertilization for couples at high risk for genetic conditions for which the disease-causing variant(s) has already been identified in the parent(s). Determining the genotype of the embryo from a single cell allows selection of unaffected embryos to implant.

Prenatal diagnosis determines whether a variant previously identified in the family is present in a fetus. Specimens for analysis traditionally have been obtained by chorionic villus sampling or amniocentesis. Prenatal diagnosis can provide reassurance or guide decisions regarding options as to whether to continue or terminate a pregnancy with an affected fetus. Prenatal and preimplantation diagnosis are usually requested in very serious diseases with an AR inheritance pattern such as ARPKD or congenital NS, and diseases with X-linked inheritance such as XLAS or Fabry disease. On the other hand, for AD diseases with adult onset, the demand is very low.

Genetic testing can play an essential role in evaluating children and young adults with CKD and thus should be considered (Vivante and Hildebrandt, 2016; Aymé *et al.*, 2017). Despite of this, genetic testing in ADPKD patients still has controversy. In addition, approximately 80% of pediatrics patients with idiopathic NS respond to glucocorticoids and are not of genetic cause (Benoit, Machuca and Antignac, 2010). Some recommendations for genetic testing in these two diseases are exposed:

Genetic testing for ADPKD is usually not done because of the clearly established imaging diagnostic criteria and the technical challenges of sequencing *PKD1*. However, the availability of disease modifying drugs like tolvaptan have increased the utility of genetic testing in presymptomatic individuals. There is wide variation in clinical practice facing children and young adults with confirmed or a family history of ADPKD with regard genetic counseling and testing. Generally, genetic testing is not recommended in minors at-risk of ADPKD since it might does not have therapeutic consequences until adulthood. However, whilst ADPKD has traditionally been thought of as an adult disease, with established renal failure tending to occur in or after the 6th decade, there is clear evidence of earlier manifestation in children and young adults. In fact, approximately 3% of children who carry ADPKD-disease causing variants have either very-early onset or unusually rapid progressive disease (Boyer *et al.*, 2007; Audrézet *et al.*, 2016). Genetic testing for ADPKD patients is indicated in a number of scenarios: (i) early and severe cases, since in this cases hypomorphic alleles and/or oligogenic inheritance could be involved (Rossetti *et al.*, 2009; Bergmann *et al.*, 2011; Harris and Hopp, 2013); (ii) in patients with a negative family history of ADPKD, (iii) marked intrafamilial disease variability, or (iv) atypical renal imaging, because of a potential phenotypic overlap with several other cystic kidney diseases; (v) when a definite diagnosis is required in young individuals, such as a potential living related donor in an affected family with equivocal imaging data; (vi) in patients requesting genetic counseling, especially in couples who desire preimplantation genetic testing (Trujillano *et al.*, 2014; Bergmann *et al.*, 2018; Lanktree *et al.*, 2019). All patients or families displaying one of these scenarios should be referred to specialized centers for further testing.

International Pediatric Nephrology Association (IPNA) developed comprehensive clinical practice recommendations on the diagnosis and management of SRNS in children (Trautmann *et al.*, 2020). They recommend genetic testing to be performed in all children diagnosed with primary SRNS. Early identification of genetic forms of SRNS is important as these patients are unlikely to benefit from prolonged and potentially harmful immunosuppression, may avoid the

necessity of a renal biopsy, and inform a low risk of post-transplant recurrence. In low-resource settings, they suggested giving priority to those patients with a likely genetic cause of the disease: familial cases (family history of proteinuria/hematuria or CKD of unknown origin), cases with extrarenal manifestations; and also to those with undergoing preparation for renal transplantation. In addition, they suggested performing genetic testing before a kidney biopsy and avoiding kidney biopsy in patients with familial and/or syndromic cases or genetic causes of SRNS. Furthermore, they did not recommend performing genetic testing in patients with initial steroid sensitivity that subsequently developed steroid resistance later in their disease course (i.e., secondary steroid resistance).

At present, clinicians are generally advised to start with a disease-specific gene panel and if the results are negative, to proceed to a Mendeliome panel (NGS approach targeting all genes which have been previously linked to single-gene disorders), WES, or even whole genome sequencing (WGS).

4. GENETIC DIAGNOSIS OF INHERITED KIDNEY DISEASE

Genetic diagnosis aims to identify the disease-causing variant(s) in the gene that cause a disease in an individual patient, but the wealth of variation within human genome makes this task difficult. The human genome contains approximately 3 billion DNA nucleotides, of which ~20 million may be altered without major consequences for an individual's health (Auton *et al.*, 2015). It harbors ~20,000 genes, of which approximately 4,521 have been implicated in human disease (https://www.omim.org/statistics/geneMap). Variation in genetic sequence includes:

- Single nucleotide variants (SNVs): substitution of a single base.
- Small insertion-deletion (INDELs): small insertion or deletion, usually from one to 49 base pairs.
- Copy number variation (CNV): duplication or deletion of genomic regions larger than 50 base pairs (Alkan, Coe and Eichler, 2011).
- Chromosomal imbalance rearrangements: deletions and duplications of entire chromosomes or segments of chromosomes. Inversions and translocations can also occur as a result of genome breakage followed by a rejoining of the broken ends in a different order that the original one.

A precise clinical diagnosis of nephropathies is generally complicated due to their high phenotypic overlap and huge genetic heterogeneity, with more than 400 known causative genes identified so far. The number of genes associated with IKD is constantly growing as the use of massively parallel sequencing expands. Thus, a major challenge in genetic diagnostics is to identify which variants are disease-causing variants.

4.1. GENETIC TESTING MODALITIES

Common modalities for genetic diagnosis include Sanger sequencing, chromosomal microarrays analysis (CMA), and NGS approaches, which include gene panels, WES, and WGS (Table 2).

Table 2. Major genetic testing modalities. Adapted from (Cocchi and Nestor, 2020)

Modality	Primary Scope	Advantages	Disadvantages	Uses
Sanger sequencing	Identification of SNVs and small INDELs within a DNA segment of <1 kb	• High analytical accuracy • Easier and faster sequence interpretation compared with NGS • No risk of secondary findings	• Limited resolution (<1 kb; cannot detect large structural variants • Increasingly time- and cost-inefficient with increasing gene length and/or number of genes tested	• Confirmation of NGS findings • Regions refractory to NGS, such as GC-rich, highly repetitive segments • Patients whose phenotype is indicative of a disorder caused by variants in one specific small gene
Chromosomal microarrays	Identification of small chromosomal rearrangements/CNVs	• Higher resolution enables detection of CNVs missed by karyotyping • Genome-wide analysis	• Cannot detect SNVs, INDELs, and small CNVs • Limited ability to detect balanced chromosomal rearrangements, low-grade somatic mosaicism, and CNVs in certain regions (such as pseudogenes and repetitive regions)	•Patients with phenotypes strongly suggestive of large rearrangements, such as multiple congenital anomalies and developmental diseases
Targeted gene panels	Identification of SNVs/INDELs within genes of interest for the clinical phenotype	• Can be optimized to ensure high coverage of the targeted regions allowing detection of CNVs, complex genomic regions, and mosaicism • Interrogation of genes that are related to the clinical indication	• Testing a limited number of genes decreases diagnostic sensitivity, especially for genetically and/or phenotypically heterogeneous disorders • Challenges of panel design (gene selection and need for frequent updates)	• Patients with specific disorders • Patients with nonspecific phenotypes and CKD of unknown origin.

		facilitates interpretation and minimizes risk of secondary findings	• Low capacity for sequence reanalysis	
Whole exome sequencing (WES)	Identification SNVs/INDELs within coding regions of the genome	• Interrogation of the coding regions • Genome-wide scope enables sequence reanalysis and discovery of novel genes	• Lower analytical sensitivity and specificity than WGS owing to limited coverage of certain regions and inability to accurately call certain types of variants (such as INDELs) • Can lead to multiple candidate variants, increasing time required for interpretation and need for follow-up testing • Burden of secondary findings in genes unrelated to the primary indication for testing	• Patients with unspecific phenotype or with CKD of unknown origin. • Patients left undiagnosed by targeted gene panels
Whole genome sequencing (WGS)	• Identification of SNVs/INDELs/ CNVs within coding and non-coding regions of the genome	• Superior diagnostic and analytical sensitivity to WES owing to its ability to assess SNVs, INDELs, and CNVs in coding and non-coding regions and more complete per-base coverage	• Difficulty of interpreting non-coding variants • Large amount of data generated results in substantial time and monetary costs, hindering return of results • Burden of secondary findings in genes unrelated to the primary indication for testing • Burden of long-term sequence data storage	• Patients with unspecific phenotype or with CKD of unknown origin. • Patients left undiagnosed by all other genetic testing modalities

Abbreviations: CNVs, copy number variations; INDELs, insertions/deletions; NGS, next generation sequencing; SNVs, single nucleotide variants; WES, whole exoma sequencing; WGS, whole genome sequencing.

4.1.1. Sanger sequencing

Sanger sequencing is the classic DNA sequencing method. It is a very sensitive method for the identification of SNVs and INDELs, but in each experiment it only allows to sequence a single DNA fragment with a maximum length of about 1,000 nucleotides. This methodology is very laborious for diseases caused by genes with a large number of exons (e.g. *PKHD1* gene with 67 exons) and/or diseases with genetic heterogeneity (with multiple causal genes). Currently, Sanger sequencing remains the gold standard for molecular diagnosis in certain cases:

a) When a single-gene disorder is suspected and is caused by a gene divided in a few exons. For instance, Fabry disease caused by the *GLA* gene, which is divided in only 7 exons.

b) Screening at-risk family members for a known disease-causing variant.

c) Confirm the results obtained by NGS approaches.

d) Sequencing specific genes or regions that are not attainable with NGS approaches.

In other cases, it becomes increasingly costly and time-inefficiently, limiting its utility for genetically heterogeneous conditions. In addition, Sanger sequencing cannot detect larger structural variants and must be complemented with the so-called multiplex ligation-dependent probe amplification (MLPA) technique, which allows the detection of deletions and duplications of an exon, multiple exons or a complete gene.

4.1.2. Next Generation Sequencing

NGS technology, also called Massively Parallel Sequencing, is the most widely used approach for genetic diagnosis of IKD. It allows for simultaneous sequencing of selected regions of the genome, at a relatively low cost. Selected regions can be multiple genes of interest (targeted gene panel), all genome protein coding-regions (WES), of both coding and non-coding regions of the whole genome (WGS). Furthermore, NGS allows the detection of all types of genetic variants, from single nucleotide variants to CNVs, although the sensitivity for the detection of CNVs is low and sophisticated bioinformatic tools are necessary to detect them. In some cases, an orthogonal validation method to confirm or discard the identified variant might be needed. For example, SNVs and INDELs can be validated by Sanger sequencing, and CNVs can be validated using aCGH or MLPA.

4.1.2.1.   Targeted gene panels

This approach targets coding regions of a selected set of genes associated with a specific disease or group of diseases. NGS gene panels use targeted enrichment of selected genes to provide rapid and inexpensive sequencing at higher coverage than that achieved with WES or WGS. Gene panel are the first-line diagnostic test in many laboratories. Some panels contain the specific genes for a certain phenotype (Bullich *et al.*, 2018) and other panels can be very broad and contain the genes responsible for all IKD (Mansilla *et al.*, 2019).

The diagnostic efficiency of specific gene panel depends entirely on the panel design and the depth of coverage at which it is sequenced. Important advantages of limiting the number of

genes sequenced are that it lowers the chance to identify incidental findings in genes unrelated to the primary indication for testing and reduce the number of variants to be assessed. However, the genes included in a specific panel must be periodically updated as new genes are discovered. In addition, it has low capacity of sequence reanalysis. For that reason, if the targeted panel testing is negative, the clinician can select another panel with broader content or proceed directly to WES or WGS. This sequential procedure may be the most comprehensive and cost-effective approach, particularly among patients whose presentation is strongly suggestive of a specific category of genetic disease (Xue *et al.*, 2015; Ars and Torra, 2017).

4.1.2.2. Whole Exome Sequencing

This sequencing approach examines nearly all coding regions of the genome (~20,000 genes)(Patwardhan *et al.*, 2015). WES has been used in many laboratories as a second-line diagnostic test when a targeted gene panel has not identified any monogenic cause of the disease or as a first-line diagnostic test in patients with atypical or nonspecific phenotypes. Virtual panels of candidate genes are usually analyzed from the data obtained from WES. If the analysis of the virtual panel does not identify a pathogenic variant that explains the disease, the WES data can be reanalyzed to try to identify pathogenic variants in genes that were not in the initial panel.

Important advantages of WES are that it allows detecting disease-causing variants in the vast majority of genes in the genome, enables sequencing reanalysis of the sequencing data and the discovery of novel candidate genes not previously described in the literature. However, the coverage per base is generally lower than with targeted gene panels, and can result in suboptimal coverage of some relevant genes (e.g., *GREB1L*). It has limited sensitivity of complex genomic regions that may be clinically relevant (e.g. the duplicated region of the *PKD1* gene with 6 pseudogenes, regions rich in GCs), higher probability of identifying incidental findings, and limited reliability for INDELs and CNVs. Furthermore, it has a large number of variants to interpret, increasing time required for interpretation and the data to store.

4.1.2.3. Whole Genome Sequencing

WGS analyzes the entire genome (exons and introns) of all genes in the genome, as well as intergenic regions. It enables the detection of splicing or regulatory variants with large phenotypic effect, although this is at the present time limited by the incomplete

understanding of the function of the most non-coding regions. WGS is generally performed for research purposes or for very complex cases where WES has been negative and there is a very clear suspicion that the cause of the disease is genetic. It has the advantage that it has superior diagnostic and analytical sensitivity owing to its ability to assess for SNVs, INDELs, and CNVs in coding and non-coding regions, and can detect certain chromosome rearrangements that cannot be detected either with panels or with WES. Similar to WES, it allows for future reanalysis of the data. Its potential drawbacks include the relatively high cost of the test (although this is progressively decreasing), the difficulty of interpreting non-coding variants, the possibility of detecting incidental findings, the large number of variants to interpret, increasing time required for interpretation and the data to store.

4.1.3. Chromosomal Microarray Analysis

CMA enables the detection of microdeletions/duplications and represents an important improvement over classical karyotyping. While karyotyping provides a whole genome analysis by visual inspection of every chromosome (number and structure), its resolution is limited to what you can see under the microscope. This includes all aneuploidies, structural rearrangements, large deletions, and large duplications, but chromosomal imbalances smaller than 5 to 7 million base pairs (5-7 Mb) are considered to be beyond its the detection limit. These submicroscopic imbalances usually do not contain important coding regions. However, certain CNVs are associated with genetic conditions that cause birth defects and/or intellectual disability. Hallmark examples include 17q12 deletion, 16p11.2 and 22q11.2 microdeletions. On the other hand, CMA is capable to identify both small and large CNVs (Schaaf, Wiszniewska and Beaudet, 2011). Thus, this is the test of choice when a structural variant is suspected in a patient, such in patients with syndromic CAKUT, in which CMA has shown to be an effective first-line test (Weber *et al.*, 2011; Sanna-cherchi *et al.*, 2012; Verbitsky *et al.*, 2019).

Unlike karyotyping, CMA cannot detect balanced chromosomal rearrangements (do not cause a net loss or gain of genetic material), such as balanced chromosomal translocations, inversions and uniparental disomies. It has also limited sensitivity to detect mosaicism and changes in certain regions such as pseudogenes or repetitive elements. It can be performed either by Microarray-based Comparative Genomic Hybridization (aCGH) or by using a SNP array. Both of these techniques offer excellent genome coverage and use enrichment of probes in clinically relevant regions.

4.2. CLASSIFICATION OF THE PATHOGENICITY OF THE VARIANTS

NGS provides a wealth of data on genetic variation. The complexity of data processing, analysis and interpretation requires the development of appropriate bioinformatical tools. Thus, variant filtering and prioritization strategies are used to determine the disease-causing variant or variants among the rest of the identified variants by NGS in one individual. This process is one of the most relevant tasks in genetic diagnosis and allows defining a precise etiological diagnosis that can be decisive in the management of the patient.

Evaluating the pathogenicity of variants is challenging. It can be difficult to discern whether genetic variants are disease-causing or not. To facilitate variant interpretation, data sharing in the form of disease-specific variant databases such as ClinVar (www.ncbi.nlm.nih.gov/clinvar), the Human Gene Mutation database (HGMD) (www.hgmd.org), and the Leiden Open Access Variation database (LOVD) (www.lovd.nl) is very helpful. It is currently well recognized that variants reported as disease-causing years ago are often subsequently downgraded if reanalyzed (Manrai, Ioannidis and Kohane, 2016). The development of large public population databases such as Genome Aggregation Database (gnomAD) has shed light on the spectrum of allele frequencies across populations and has demonstrated that a large number of previously reported variants are unlikely to be pathogenic because they are present at frequencies exceeding the prevalence of the associated disease.

The publication in 2015 of the American College of Medical Genetics and the Association for Molecular Pathology (ACMG/AMP) guidelines for the interpretation and systematic classification of sequence variants based on their probability of causing a particular monogenic disease has helped genetitians and clinicians to interpret the identified variants (Richards *et al.*, 2015). These recommendations are now widely used in genetic diagnostic laboratories around the world. However, this guidance did not intend to fulfill the needs of the research community to identify new genes in disease.

These guidelines divided sequence variants into 5 classes in accordance with IARC recommendations (Plon *et al.*, 2008):

- Class I: benign
- Class II: likely benign
- Class III: variant of uncertain significance (VUS)
- Class IV: likely pathogenic
- Class V: pathogenic

Variants that are classified as "pathogenic" and "likely pathogenic" are considered to be disease-causing. Class I, II, and III have no diagnostic value. However, VUS can be reported in a genetic diagnosis report mainly when no pathogenic/likely pathogenic variant has been identified and if they are located in a gene that has been associated with the phenotype presented by the patient. Many of these VUS variants are being reclassified as probably pathogenic or probably benign as more evidence becomes available. As the term "likely" could have a wide range of uses and easily create confusion, they proposed its use to mean greater than 90% certainly of a variant either being pathogenic or benign.

To classify the variants into one of the five classes, the ACMG/AMP guidelines evaluate different evidence criteria (Figure 9):

1) Characteristics inherent to the variant. Certain types of variants (e.g., nonsense, frameshift, canonical splice sites, initiation of a codon, single exone or multiexone deletion) can often be assumed to disrupt gene function. If the variant is located in a mutational hot spot and/or critical and well-established functional domain.

2) If the same amino acid change has been previously established as a pathogenic variant regardless of nucleotide change. It can also be assumed to be pathogenic, unless the variant acts directly through the specific DNA change instead of through the amino acid change, in which case the assumption of pathogenicity may no longer be valid.

3) Frequency of the variant in reference population databases. Pathogenic variants generally do not appear in these population databases or have extremely low population frequencies.

4) If there are well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product.

5) Pathogenicity predictions of amino acid change variants (missense) obtained with multiple bioinformatic algorithms available online (e.g., *BayesDel_addAF, DANN, DEOGEN2, EIGEN, FATHMM-MKL, LIST-S2, M-CAP, MVP, MutationAssessor, MutationTaster, SIFT*) that assess the probability that a variant is pathogenic or not.

6) Genotype-phenotype correlation, if the phenotype or family history of the patient correlates with the clinical manifestations attributed to the gene that presents the candidate variant.

7) Gene inheritance pattern, p. ex. if the gene is recessive, another candidate variant must exist in the same gene *in trans* (in the other chromatin chain).

8) Family segregation of the variant: if the variant is located in an AD gene, the affected relatives present the variant and the healthy ones do not. If the patient is a *de novo* case of the disease and the parents do not have the variant.

9) Scientific articles in which the variant is described with functional data indicating an alteration of the resulting protein and/or clinical information of the patients in which the candidate variant has been identified.

10) Genotype-phenotype correlation databases such as ClinVar or HGMD or of specific genes such as the PKD Mayo database for the *PKD1* and *PKD2* genes; the LOVD for the *COL4A3* and *COL4A4* genes, which generally includes the clinical interpretation of the variant, if it has been described in the scientific literature.

These criteria are combined according to scoring rules that allow classification of each variant in one of the 5 categories mentioned above (Richards *et al.*, 2015).

	Benign		Pathogenic			
	Strong	**Supporting**	**Supporting**	**Moderate**	**Strong**	**Very strong**
Population data	MAF is too high for disorder BA1/BS1 **OR** observation in controls inconsistent with disease penetrance BS2			Absent in population databases PM2	Prevalence in affecteds statistically increased over controls PS4	
Computational and predictive data		Multiple lines of computational evidence suggest no impact on gene /gene product BP4 Missense in gene where only truncating cause disease BP1 Silent variant with non predicted splice impact BP7 In-frame indels in repeat w/out known function BP3	Multiple lines of computational evidence support a deleterious effect on the gene /gene product PP3	Novel missense change at an amino acid residue where a different pathogenic missense change has been seen before PM5 Protein length changing variant PM4	Same amino acid change as an established pathogenic variant PS1	Predicted null variant in a gene where LOF is a known mechanism of disease PVS1
Functional data	Well-established functional studies show no deleterious effect BS3		Missense in gene with low rate of benign missense variants and path. missenses common PP2	Mutational hot spot or well-studied functional domain without benign variation PM1	Well-established functional studies show a deleterious effect PS3	
Segregation data	Nonsegregation with disease BS4		Cosegregation with disease in multiple affected family members PP1	Increased segregation data →		
De novo data				De novo (without paternity & maternity confirmed) PM6	De novo (paternity and maternity confirmed) PS2	
Allelic data		Observed in *trans* with a dominant variant BP2 Observed in *cis* with a pathogenic variant BP2		For recessive disorders, detected in trans with a pathogenic variant PM3		
Other database		Reputable source w/out shared data = benign BP6	Reputable source = pathogenic PP5			
Other data		Found in case with an alternate cause BP5	Patient's phenotype or FH highly specific for gene PP4			

Figure 9. ACMG/AMP criteria for variant interpretation (Richards *et al.*, 2015)**.** BS, benign strong; BP, benign supporting; FH, family history; LOF, loss of function; MAF, minor allele frequency; path, pathogenic; PM, pathogenic moderate; PP, pathogenic supporting; PS, pathogenic strong; PVS, pathogenic very strong.

4.3. OPPORTUNISTIC GENOMIC SCREENING

When a genetic testing is performed, especially in WES and WGS, variants irrelevant to the clinical question but with medical relevance for the health prospects and/or reproductive choices of the patient or patient's family can be identified. Different nomenclatures have been used to name these variants, including 'incidental findings', 'accidental findings', 'unsought for findings', 'co-incidental findings', and 'unsolicited findings'. Conceptually, 'incidental findings' and 'secondary findings' have different meaning and need to be distinguished. Notwithstanding both terms refer to results not related to the original reason for testing; the first ones are not actively sought for whereas the second ones are.

Debate is ongoing about if clinical diagnostic laboratories performing NGS should or should not actively look for additional variants unrelated to the initial purpose of testing, which however could be of medical relevance. Some organizations such as European Society of Human Genetics (ESHG) recommended genome analysis to be restricted to the original test indication and as targeted as possible (Van El *et al.*, 2013; de Wert *et al.*, 2021). On the contrary, other organizations such as ACMG recommended a routine analysis and deliberate search of pathogenic and likely pathogenic variants in a predefined set of 73 'actionable' genes regardless of patient age, if the patient or family agrees (Miller *et al.*, 2021). ACMG uses the term 'opportunistic screening' for this deliberate search for secondary findings in the context of genome sequencing in health care.

Access to active search for actionable secondary findings in diagnostic practice is a major psychological and ethical issue for genomic medicine, especially in children. The benefits of informing children or their families of secondary findings, which usually do not cause disease until adulthood, are unclear. A quantitative analysis of 513 parents of children with undiagnosed developmental disorders showed that they wished to receive exhaustive information when WES is performed, including secondary findings (Chassagne *et al.*, 2019). A published meta-analysis of studies examining hypothetical secondary findings preferences for WES/WGS providers and recipients shown the overwhelming majority believed that some form of secondary findings should be returned if identified (Mackley *et al.*, 2017). Potential WES/WGS recipients' views were largely influenced by a sense of rights, whereas views of genomics professionals were informed by a sense of professional responsibility. Of note, many of the analyzed studies reported a small proportion of recipients who wanted only primary findings, and clinicians with genetic knowledge were found slightly less support for returning secondary findings. On contrary, presymptomatic diagnostic surveys of families at risk of carrying a genetic predisposition to breast cancer or heart disease revealed a much lower

percentage of patients willing to undergo additional genetic testing when the molecular basis of their condition has been identified (Ropka *et al.*, 2006; Christiaans *et al.*, 2008)

5. PERSONALIZED MEDICINE

Personalized medicine, also known as precision medicine, is an emerging practice of medicine that uses an individual's genetic profile to guide decisions made in regard to the prevention, diagnosis, and treatment of disease. The significance of identifying the genetic cause is multifold. It can provide an accurate diagnosis, avoid the need of invasive diagnostic procedure such as kidney biopsies, allow for genetic counseling and screening of at-risk family members, and facilitate personalized treatment and prognostication.

Potential therapy is available for some rare causes of SRNS. For instance, patients with disease-causing variants identified in a gene encoding enzymes of the CoQ10 biosynthesis (*COQ2*, *COQ6*, *ADCK4* or *PDSS2*) may respond to coenzyme Q_{10}, offering a cheap and innocuous treatment, since partial response to this treatment has been described (Montini, Malaventura and Salviati, 2008; Heeringa *et al.*, 2011; Ashraf *et al.*, 2013). Similarly, individuals with *TRPC6* disease-causing variants may potentially be amenable to treatment with calcineurin inhibitors (Schlöndorff *et al.*, 2009), patients with Imerslund-Gräsbeck syndrome carrying *CUBN* disease-causing variants may be amenable to treatment with vitamin B_{12} (Gräsbeck, 2006).

Over the past two decades research into X-linked hypophosphatemia (XLH) mechanisms has generated novel potential targets for treatment. Recent approval of burosumab for clinical use in patients with XLH represented a substantial advancement in management of these patients. XLH is the most common genetic form of hypophosphatemic rickets and osteomalacia. It is caused by disease-causing variants in the *PHEX* gene situated on the chromosome X, which results in elevated circulating levels of hormone fibroblast growth factor 23 (FGF23) leading to impaired renal reabsorption of phosphorus, hypophosphatemia, and impaired activation of vitamin D (Carpenter *et al.*, 2011; Imel and Econs, 2012).

Another IKD with available treatment is Fabry disease. Fabry disease is a rare and progressive X-linked lysosomal disorder caused by pathogenic variants in the *GLA* gene, resulting in functional deficiency of α-galactosidase A (α-Gal A). The deficiency causes accumulation of glycosphingolipid substrates within lysosomes in various tissues, producing impairment of kidney, heart, and brain function and to early death (Mehta *et al.*, 2010). Intravenous enzyme replacement therapy has been the current standard treatment for Fabry disease. Migalastat (1-deoxygalactonojirimycin, AT1001), is a pharmacological chaperone that binds to and

stabilizes amenable mutant forms of α-Gal A, facilitating lysosomal trafficking and increasing lysosomal enzyme activity. This chaperone-based therapy has been developed for Fabry disease as an alternative to intravenous enzyme replacement therapy in patients with amenable variants (Benjamin *et al.*, 2017).

The only therapeutic drug for patients with ADPKD approved so far is tolvaptan. It is a potent, highly selective, orally available, vasopressin receptor antagonist that has shown to slow the growth of cysts and slow down the deterioration of renal function (Torres *et al.*, 2012). The complexity of selecting the appropriate patients for treatment is still a major challenge for clinicians. In general, only patients with a high risk of reaching KF at a relatively early age are suitable candidates for tolvaptan treatment. A recent study showed that patients with ADPKD and no *PKD1/2* disease-causing variant identified had less improvement with tolvaptan than patients with identified molecular cause (Sekine *et al.*, 2020). Also, the use of the PROPKD score (containing the genotype) has been used to predict disease progression (Cornec-Le Gall *et al.*, 2016). Thus, detecting disease-causing variants in *PKD1* and *PKD2* may be useful for predicting the effectiveness of tolvaptan.

Genetics can also affect the development and design of clinical trials. The incorporation of genetic analysis in clinical trials can identify a subset of patients who may benefit most from the therapy. For instance, different trials are ongoing for AS since it is a very attractive disease for pharmaceutical companies to target because there is no curative treatment, the number of patients to be treated is substantial, and it could be an excellent model of CKD with proteinuria and fibrosis that may be extrapolated to other causes of CKD (Torra and Furlano, 2019).

Finally, pharmacogenomic analyses can help identify variants that affect drug absorption or metabolism, enabling better assessment of dosage, safety, and side effects. Despite its promise, pharmacogenetics is still in its early stages, particularly in the setting of kidney disease.

AIMS

This PhD thesis has been carried out with the aim of increasing the knowledge on clinical and genetic heterogeneity of IKD, with special emphasis on patients with early-onset CKD (<30 years of age). More specifically, the aims were:

1. To improve the genetic diagnostics of IKD by increasing its diagnostic yield and widening the range of diagnosed nephropathies as well as obtaining more complete genetic information.
2. To identify the genes with the highest likelihood of bearing disease-causing variants in children and young patients with CKD.
3. To determine the clinical utility of genetic testing in patients with early-onset CKD.
4. To elucidate the genetic cause of disease in patients with atypical early-onset CKD.
5. To study at clinical and genetic level a cohort of patients with proteinuria-causing variants in the *CUBN* gene.

RESULTS

ARTICLE I: Clinical utility of genetic testing in early-onset kidney disease: seven genes are the main players

Andrea Domingo-Gallego, Marc Pybus, Gemma Bullich, Mónica Furlano, Laia Ejarque-Vila, Laura Lorente-Grandoso, Patricia Ruiz, Gloria Fraga, Mercedes López González, Juan Alberto Piñero-Fernández, Lidia Rodríguez-Peña, Isabel Llano-Rivas, Raquel Sáez, Anna Bujons-Tur, Gema Ariceta, Lluis Guirado, Roser Torra* & Elisabet Ars*

*These authors contributed equally to this work.

SUMMARY

Background: Inherited kidney diseases are one of the leading causes of chronic kidney disease (CKD) that manifests before the age of 30 years. Precise clinical diagnosis of early-onset CKD is complicated due to the high phenotypic overlap, but genetic testing is a powerful diagnostic tool.

Aims: The aims of this study were to develop a genetic testing strategy to maximize the diagnostic yield for patients presenting with early-onset CKD and to determine the prevalence of the main causative genes.

Methods: We performed genetic testing of 460 patients with early-onset CKD of suspected monogenic cause using next-generation sequencing of a custom-designed kidney disease gene panel in addition to targeted screening for c.428dupC *MUC1*.

Results: We achieved a global diagnostic yield of 65% (300/460), which varied depending on the clinical diagnostic group: 77% in cystic kidney diseases, 76% in tubulopathies, 67% in autosomal dominant tubulointerstitial kidney disease, 61% in glomerulopathies, and 38% in congenital anomalies of the kidney and urinary tract. Among the 300 genetically diagnosed patients, the clinical diagnosis was confirmed in 77%, a specific diagnosis within a clinical diagnostic group was identified in 15%, and 7% of cases were reclassified. Of the 64 causative genes identified in our cohort, seven (*COL4A3, COL4A4, COL4A5, HNF1B, PKD1, PKD2*, and *PKHD1*) accounted for 66% (198/300) of the genetically diagnosed patients.

Conclusions: Two-thirds of patients with early-onset CKD in this cohort had a genetic cause. Just seven genes were responsible for the majority of diagnoses. Establishing a genetic diagnosis is crucial to define the precise etiology of CKD, which allows accurate genetic counseling and improved patient management.

ARTICLE II: Novel homozygous *OSGEP* gene pathogenic variants in two unrelated patients with Galloway-Mowat syndrome: case report and review of the literature

Andrea Domingo-Gallego, Mónica Furlano, Marc Pybus, Daniel Barraca, Ana Belén Martínez, Emiliano Mora Muñoz, Roser Torra & Elisabet Ars

SUMMARY

Background: Galloway-Mowat syndrome (GAMOS) is a rare autosomal recessive disorder characterized by early-onset nephrotic syndrome and microcephaly with brain anomalies. *WDR73* pathogenic variants were described as the first genetic cause of GAMOS and, very recently, four novel causative genes, *OSGEP, LAGE3, TP53RK*, and *TPRKB*, have been identified.

Case presentation: We present the clinical and genetic characteristics of two unrelated infants with clinical suspicion of GAMOS who were born from consanguineous parents. Both patients showed a similar clinical presentation, with early-onset nephrotic syndrome, microcephaly, brain atrophy, developmental delay, axial hypotonia, and early fatality. We identified two novel likely disease-causing variants in the *OSGEP* gene. These two cases, in conjunction with the findings of a literature review, indicate that *OSGEP* pathogenic variants are associated with an earlier onset of nephrotic syndrome and shorter life expectancy than *WDR73* pathogenic variants.

Conclusions: Our findings expand the spectrum of pathogenic variants in the *OSGEP* gene and, taken in conjunction with the results of the literature review, suggest that the *OSGEP* gene should be considered the main known monogenic cause of GAMOS. Early genetic diagnosis of GAMOS is of paramount importance for genetic counseling and family planning.

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Novel homozygous *OSGEP* gene pathogenic variants in two unrelated patients with Galloway-Mowat syndrome: case report and review of the literature

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Abstract

Background: Galloway-Mowat syndrome (GAMOS) is a rare autosomal recessive disorder characterized by early-onset nephrotic syndrome and microcephaly with brain anomalies. *WDR73* pathogenic variants were described as the first genetic cause of GAMOS and, very recently, four novel causative genes, *OSGEP*, *LAGE3*, *TP53RK*, and *TPRKB*, have been identified.

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Conclusions: Our findings expand the spectrum of pathogenic variants in the *OSGEP* gene and, taken in conjunction with the results of the literature review, suggest that the *OSGEP* gene should be considered the main known monogenic cause of GAMOS. Early genetic diagnosis of GAMOS is of paramount importance for genetic counseling and family planning.

Keywords: Galloway-Mowat syndrome, Nephrotic syndrome, *OSGEP*, KEOPS complex, Genetic testing, Case report

Background

Galloway-Mowat syndrome (GAMOS) (OMIM #251300) is a rare autosomal recessive syndrome first described in 1968. It is a clinically heterogeneous condition characterized by early-onset nephrotic syndrome associated with microcephaly, gyral abnormalities of the brain, and delayed psychomotor development. Most patients also present dysmorphic facial features, including hypertelorism, ear abnormalities, and micrognathia. Most affected individuals die in early childhood [1, 2]. The estimated prevalence is < 1/1,000,000 but it is likely that many cases remain misdiagnosed or undiagnosed.

Truncating variants in *WDR73* gene were described as the first monogenic cause of GAMOS in two families [3]. The protein encoded by this gene is implicated in the regulation of the microtubule network during cell cycle progression, proliferation, and survival [3–5]. Homozygous missense variants in the *WDR73* gene were later reported [6, 7]. Recently, pathogenic variants in the *OSGEP*, *LAGE3*, *TP53RK*, and *TPRKB* genes have been identified as novel monogenic causes of GAMOS. These genes encode four subunits of the evolutionary highly

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conserved KEOPS (kinase, endopeptidase, and other proteins of small size) complex. This complex plays an important role in brain and renal development. To date, the genetic cause of GAMOS has been reported in 54 families: *OSGEP* in 26 (48.15%), *WDR73* in 19 (35.18%), *TP53RK* in 4 (7.40%), *LAGE3* in 3 (5.55%), and *TPRKB* in 2 (3.70%) [2–11].

Here, we report two unrelated patients with GAMOS carrying homozygous pathogenic variants in the newly identified *OSGEP* gene.

Case presentation

Patient 1 was a first-child male born to healthy consanguineous parents from Spain with no previous family history of kidney disease (Table 1, Fig. 1A). He was born at 39.4 weeks of gestation. Birth weight was 3400 g, height was 51 cm, and head circumference was 34 cm, with no dysmorphic features. Forty-five days after birth, the patient was diagnosed as having congenital nephrotic syndrome with severe proteinuria, hypertension, and hypothyroidism. He also presented edema, hyperkalemia, hyponatremia, and hypomagnesemia. Renal ultrasound showed poor corticomedullary differentiation in the right kidney. Renal biopsy showed diffuse mesangial sclerosis,

tubular atrophy, and primitive glomeruli (Fig. 1C). The patient progressed to end-stage renal disease and required peritoneal dialysis. He also presented left eye evisceration, dry right eye, and gastroesophageal reflux. At 5 months of age, the neurological examination revealed microcephaly with a head circumference of 38.5 cm (percentile < 1, – 4.72 SD), severe psychomotor delay for his age, and axial hypotonia. Cranial magnetic resonance imaging (CMRI) revealed brain atrophy and absence of normal myelination of the brainstem, cerebellar white matter, bilateral hemispheric white matter, internal capsules, and corpus callosum as well as abnormal intensity signal in the dentate nucleus and thalamus (Fig. 1D). In view of the congenital nephrotic syndrome and microcephaly with brain anomalies, a clinical diagnosis of GAMOS was suspected. The patient presented progressive neurological deterioration and died at 8 months of age.

Patient 2 was a female infant with normal karyotype (46, XX) born to healthy consanguineous parents from Pakistan. The patient had two healthy sisters and there was no family history of kidney disease (Table 1, Fig. 2a). She was born at 40.3 weeks of gestation. Birth weight was 2940 g, height was 49 cm, and head circumference was 32 cm with signs of microcephaly. Screening for

Table 1 Clinical data of patients 1 and 2

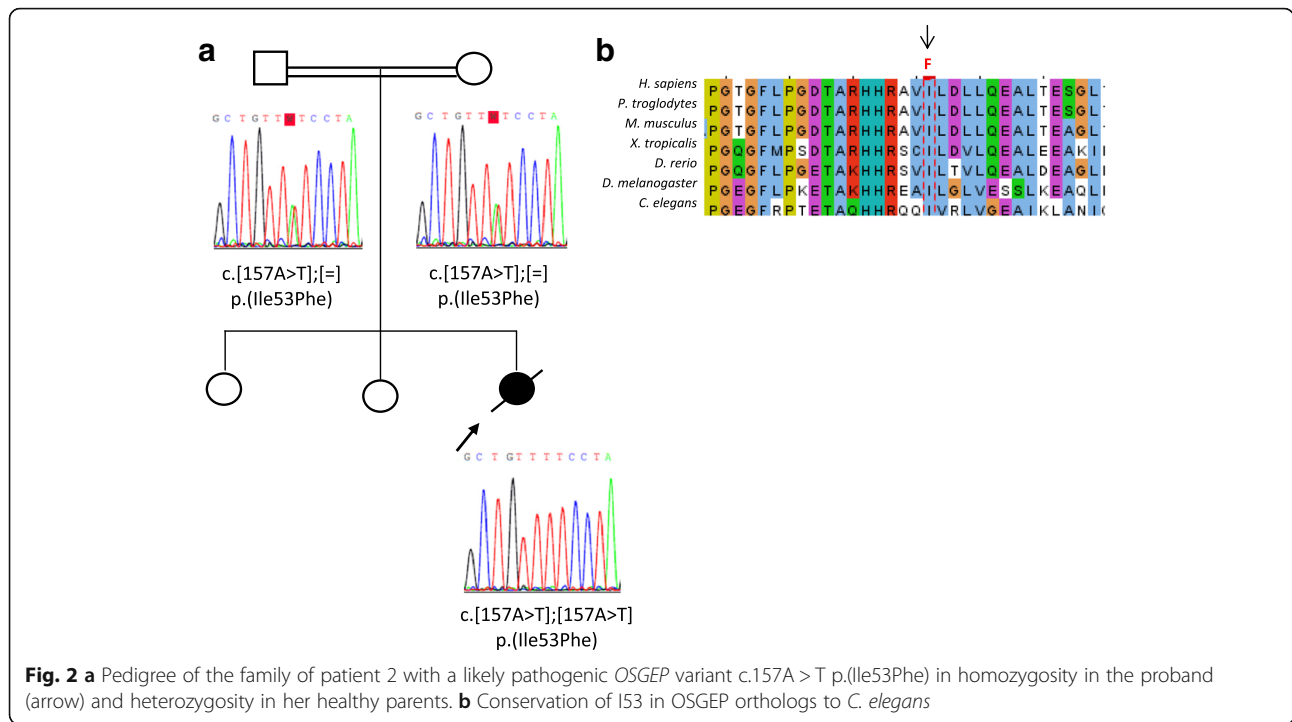| Clinical features	Patient 1	Patient 2
Sex	Male	Female
Age at death	8 months	7 months
Origin	Spanish	Pakistani
Neonatal profile (at birth)		
Gestational period (weeks)	39.4	40.3
Weight (g)	3400 (58th percentile)	2940 (17th percentile)
Height (cm)	51 (70th percentile)	49 (30th percentile)
Head circumference (cm)	34 (28th percentile)	32 (3rd percentile)
Renal phenotype		
Onset of NS (days)	45	75
Renal biopsy	DMS, tubular atrophy, primitive glomeruli	Increased glomerular mesangial matrix
Renal ultrasound	Poor corticomedullary differentiation	Cortical hyperechogenicity
Hyperkalemia	Yes	No
Hypomagnesemia	Yes	No
Neurological involvement		
Brain MRI	Microcephaly, brain atrophy, poor myelination	Microcephaly, brain atrophy
Developmental delay	Yes	Yes
Axial hypotonia	Yes	Yes
Skeletal abnormalities	No	Arachnodactyly
Dysmorphic features	No	Yes (wide nasal bridge, retrognathia, low set ears)
Others	Dry right eye Gastroesophageal reflux	Epileptiform activity Seizures

DMS Diffuse mesangial sclerosis, *NS* nephrotic syndrome
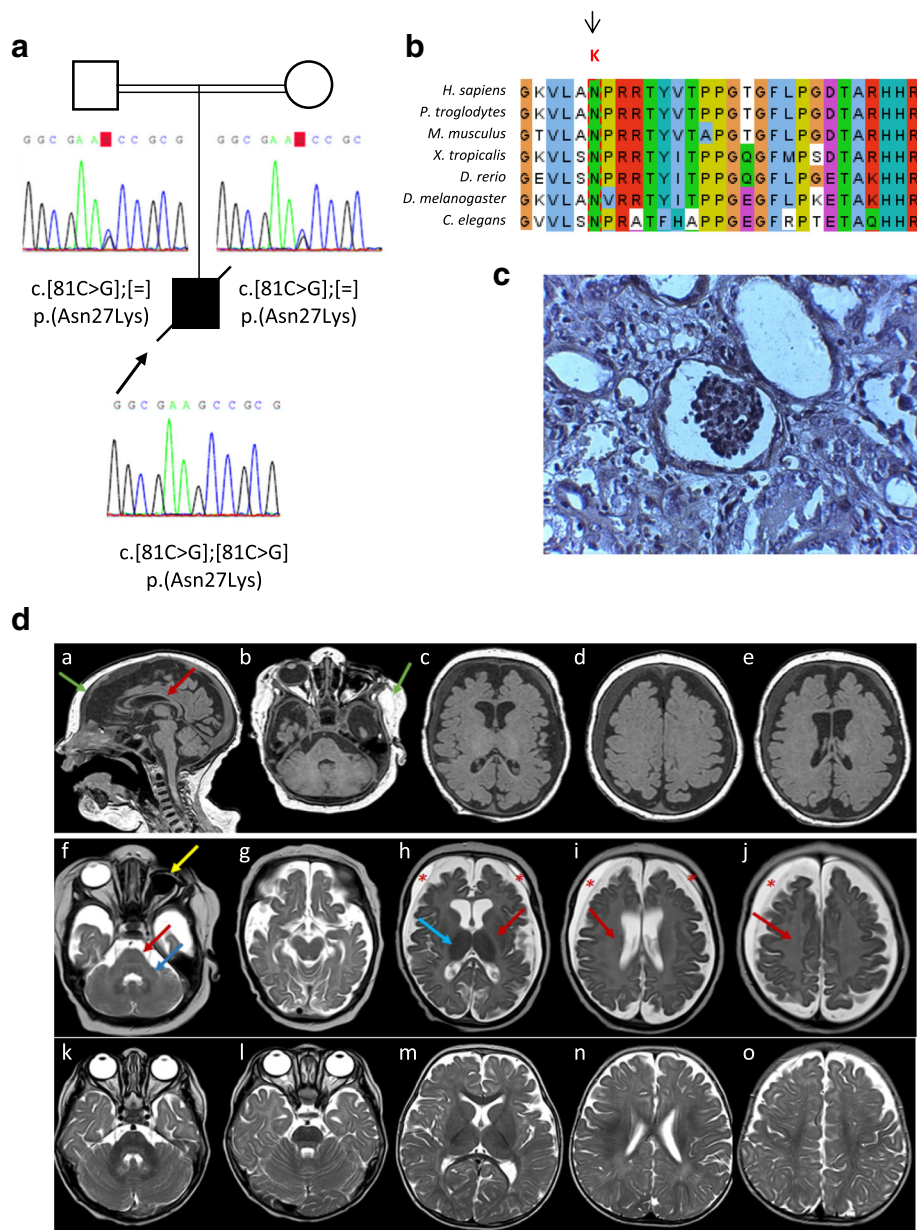
Fig. 1 a Pedigree of patient 1 with a likely pathogenic *OSGEP* variant, c.81C > G p.(Asn27Lys), in homozygosity while his consanguineous parents are healthy heterozygous carriers. **b** The identified missense variant c.81C > G p.(Asn27Lys) affects a totally conserved amino acid N27 in OSGEP orthologs. **c** Silver-stained renal biopsy from patient 1 showed glomerular collapse with mesangial matrix increase, atrophic tubules, and interstitial fibrosis on light microscopy. **d** CMRI performed in patient 1 at 8 months of age: sagittal 3 Dimensional Imaging T1 sequence (*a*), axial reconstructions (*b–e*), and axial Turbo Spin Echo (TSE) T2 (*f–j*). *k–o*: Sequence TSE T2 of normal control individual. MRI revealed craniofacial disproportion in relation to microcephaly (*a*); supratentorial cortico-subcortical atrophy with increased extra-axial space, prominence of the frontal horns, and thinning of the corpus callosum (red arrow in a); bilateral subdural frontoparietal hygromas (red asterisks in *h–j*); and atrophy of the basal ganglia (*h*). A decrease in the number and depth of the grooves was observed (*h–j*) and there was an absence of normal myelination of the brainstem (red arrow in *f*), cerebellar peduncles (blue arrow in *f*), internal capsules (red arrow in *h*), and white bihemispheric substance (arrows in *i* and *j*). Hypointense T2 signal of the thalamus was evident (blue arrow in *h*). Enucleation of the left eye is denoted by the yellow arrow in *f*. Finally, there was an increase in the thickness of the cranial and facial subcutaneous cellular tissue (green arrows in *a* and *b*)

metabolic disorders and cerebral ultrasound were normal. The patient presented dysmorphic features (wide nasal bridge, aquiline nose and retrognathia, low set ears, and arachnodactyly) with axial hypotonia and poor eye contact.

Seventy-five days after birth, she presented with nephrotic range proteinuria, hypoproteinemia with severe hypoalbuminemia, hypertriglyceridemia and hypercholesterolemia. Serum creatinine and urea were normal. Abdominal

Fig. 2 a Pedigree of the family of patient 2 with a likely pathogenic *OSGEP* variant c.157A > T p.(Ile53Phe) in homozygosity in the proband (arrow) and heterozygosity in her healthy parents. **b** Conservation of I53 in OSGEP orthologs to *C. elegans*

ultrasound showed normal-sized kidneys and correct corticomedullary differentiation with cortical hyperechogenicity, bilateral pleural effusion, and a discrete amount of fluid in the abdominal cavity. Renal biopsy showed one glomerulus with increased mesangial matrix and two normal glomeruli; fibrosis and tubular atrophy were absent. CMRI revealed severe brain atrophy with normal cerebellum and brainstem. Electroencephalogram showed normal brain activity with low-amplitude brain waves and occasional frontal left epileptiform activity. The patient evolved with failure to thrive, anemia, and electrolyte disorders and finally died from cardiorespiratory arrest in a sepsis context at 7 months of age.

Genetic study

Variant analyses of patients 1 and 2 were performed by targeted massive parallel sequencing using an updated version of our kidney disease gene panel that includes more than 200 genes causative of or associated with inherited kidney diseases (including *WDR73*, *TPRKB*, *TP53RK*, *LARGE3*, and *OSGEP* genes) [12].

Briefly, libraries were prepared according to the manufacturer's standard protocol, NimbleGen SeqCap EZ Library SR version 4.3. Patients' DNAs were fragmented and hybridized to the custom NimbleGen SeqCap EZ Choice gene panel and sequenced on a NextSeq 500 instrument (Illumina). Sequence data analysis was performed using an open-source in-house bioinformatic pipeline, as previously reported [12–14]. The mean depth of coverage per exon of *OSGEP* ranged from 153 to 433,

with 100% of the bases covered at least 100X. Prediction of pathogenicity was evaluated using different bioinformatic algorithms (DANN, GERP, dbNSFP.FATHMM, LRT, MetaLR, MetaSVM, MutationAssessor, PROVEAN, SIFT, and MutationTaster). Clinical interpretation of variants was based on American College of Medical Genetics (ACMG) recommendations [15]. All candidate pathogenic variants were validated by conventional polymerase chain reaction amplification and Sanger sequencing. Familial segregation analysis was assessed. Analysis of copy number variations (CNVs) was performed using CoNVaDING (copy number variation detection in next-generation sequencing gene panels) software [16].

Patient 1 carried a homozygous missense variant c.81C > G p.(Asn27Lys) in exon 1 of the *OSGEP* gene (NM_017807), not previously described in the literature. This variant was predicted to be pathogenic by seven prediction tools (DANN, GERP, LRT, MutationAssessor, MutationTaster, SIFT, and PROVEAN) and benign by three (dbNSFP.FATHMM, MetaLR, and MetaSVM). This variant altered an evolutionarily highly conserved residue and was absent from the population databases Genome Aggregation Database (gnomAD) and 1000 Genomes (Fig. 1B). Segregation analysis showed that both parents were heterozygous carriers of this *OSGEP* variant (Fig. 1A). We concluded that this variant was likely pathogenic (Table 2).

Patient 2 carried a homozygous missense variant c.157A > T p.(Ile53Phe) localized in exon 2 of the *OSGEP* gene. This variant has not been previously reported in

Table 2 Information of the two identified variants in the *OSGEP* gene

Patient	Variant	Population Databases			Predictor scores											Parental segregation confirmed	ACMG (Variant classification)
		dbSNP	1000G	gnomAD *f*	DANN	GERP	LRT	MA	MT	SIFT	PROVEAN	dbNSFP.FATHMM	MetaLR	MetaSVM		
Patient 1	c.81C > G p.(Asn27Lys)	No	0	0	P	P	P	P	P	P	P	B	B	B	Yes	LP (PM2, PM3, PP1, PP2, PP3, PP4)
Patient 2	c.157A > T p.(Ile53Phe)	rs780944919	0	0.0000979 (South Asians)	P	B	P	P	P	B	P	B	B	B	Yes	LP (PM2, PM3, PP1, PP2, PP4)

1000G 1000 Genomes Project, *f* Allele Frequency, *B* Benign, *gnomAD* genome Aggregation Database, *LP* Likely Pathogenic, *MA* MutationAssessor, *MT* MutationTaster, *P* Pathogenic

literature. The variant c.157A > T p.(Ile53Phe) was predicted to be pathogenic by five prediction tools (DANN, LRT, MutationAssessor, MutationTaster, and PROVEAN) and benign by five (GERP, dbNSFP.FATHMM, MetaLR, SIFT, and MetaSVM). This variant was conserved in *OSGEP* orthologs to *C. elegans* and is extremely rare in the general population (Fig. 2b), with a minor allele frequency in South Asians is 0.00009799 (3 of 30,782 sequenced alleles, no homozygous individuals) in the gnomAD database. The global allele frequency was lower than the 0.0001 threshold for recessive gene *OSGEP*. The parents were confirmed to be heterozygous carriers (Fig. 2a). We classified this variant as likely pathogenic (Table 2).

Discussion and conclusions

We report two patients who presented with nephrotic syndrome with onset at < 3 months old, primary microcephaly, and developmental delay, which are hallmarks of GAMOS. Both patients carried homozygous likely disease-causing variants in the *OSGEP* gene. This gene was recently identified as causative of GAMOS in a large cohort of 907 individuals with nephrotic syndrome [2]. Pathogenic variants in one of the four genes *TP53RK*, *TPRKB*, *LAGE3*, and *OSGEP*, encoding KEOPS complex subunits, were found in 37 out of 91 patients with GAMOS. Independently, a homozygous pathogenic variant in the *OSGEP* gene was reported in two siblings with a similar renal-neurological phenotype, also by whole exome sequencing [8].

The *OSGEP* gene encodes the *O*-sialoglycoprotein endopeptidase enzyme, which regulates the second biosynthetic step in the formation of *N*-6-threonylcarbamoyladenosine in the cytosol, essential for mRNA translational initiation and efficiency. The highly conserved KEOPS complex is implicated in several cell processes, such as control of telomere length, telomere-associated DNA damage response signaling, and genome maintenance. Zebrafish larvae knockout of the *osgep* gene resulted in primary microcephaly, with increased apoptosis in the brain compared with controls and early lethality. Knockout mouse embryos also showed microcephaly compared with wild-type embryos. Neither mutant fish nor mice showed any renal phenotype, possibly due to embryonic early lethality [2].

Great strides have been made in the understanding of GAMOS disease over the past 4 years, with the identification of its genetic bases in some patients. However, the genetic etiology of more than three-quarters of patients with a clinical diagnosis of GAMOS remains elusive, suggesting that additional causative genes remain to be identified. Currently, the principal known causative genes of GAMOS are *OSGEP* and *WDR73*.

A review of the literature based on 31 patients (26 families) bearing *OSGEP* pathogenic variants and 23 patients (13 families) with *WDR73* pathogenic variants

indicates that *OSGEP* causes earlier onset of nephrotic syndrome than *WDR73* [2–4, 6–10]. Eighty percent (25/31) of patients with *OSGEP* pathogenic variants developed nephrotic syndrome with a mean age at onset of 10.36 months (ranging onset from birth to 13 years). In comparison, 35% (8/23) of patients with *WDR73* pathogenic variants presented nephrotic syndrome at a mean age of 7.7 years (ranging from 0.5 to 16 years). Our two patients carrying *OSGEP* pathogenic variants presented with nephrotic syndrome before 3 months of age.

Renal manifestations described in GAMOS patients vary from isolated proteinuria to steroid-resistant nephrotic syndrome, and some patients even have no renal alterations during follow-up period [2–8]. Intrafamilial clinical variability has also been described in GAMOS. For instance, two siblings carrying a *WDR73* pathogenic variant manifested contrasting renal phenotype [3]. One of the affected siblings presented with nephrotic syndrome at the age of 5 years, rapidly developed chronic renal insufficiency, and died after a month, while the other had no renal symptoms at the age of 7 years [3]. A homozygous *OSGEP* pathogenic variant, c.974A > G p.(Arg325Gln), has also been associated with renal tubular anomalies [10]. It was detected in a girl with magnesium-wasting tubulopathy and partial Fanconi syndrome with a normal glomerular filtration rate who never developed nephrotic syndrome [10]. Interestingly, this variant was previously identified in two siblings with severe hypomagnesemia, hypercalciuria, and proteinuria but normal albumin levels [8]. The authors raised the question of whether these patients should be considered to be affected by a different clinical entity [8, 10].

The review of the literature also indicates that patients with *OSGEP* pathogenic variants have a shorter life expectancy than those with *WDR73* pathogenic variants. Seventy-one percent (22/31) of patients with *OSGEP* pathogenic variants died at a mean age of 1.5 years (ranging from 6 weeks to 8 years). In line with these reported cases, our patients died at 8 and 7 months of age. However, seven patients with *OSGEP* pathogenic variants were alive at 13 (2), 10.5 (1), 7 (1), 3.5 (1), and 2 (1) years and at 7 (1) months [2, 10]. It should be noted that four of them carried the above-mentioned *OSGEP* variant, c.974A > G p.(Arg325Gln), associated with renal tubular anomalies [10]. Twenty-two percent (5/23) of patients carrying pathogenic variants in the *WDR73* gene died at a mean age of 8.1 years (ranging from 2.5 to 17 years).

Nearly all *OSGEP* variants reported as causative of GAMOS are missense, except for two splicing variants [2, 8]. These variants are located throughout the *OSGEP* gene. By contrast, different types of variant in *WDR73* causative of GAMOS have been reported in the literature, including nonsense (3), frameshift (3), and missense (4). No correlation seems to exist between the type or

position of the variant and particular clinical features. Identification of the causative pathogenic variant in patients 1 and 2 confirmed the initial clinical suspicion of GAMOS and allowed precise genetic counseling to their parents. In particular, it allowed prenatal diagnosis of a baby girl without GAMOS for the parents of patient 1.

In conclusion, we report two patients with GAMOS caused by *OSGEP* pathogenic variants. These two cases, in conjunction with the reported cases in the literature, add evidence that *OSGEP* pathogenic variants are the most prevalent cause of GAMOS and are associated with a more severe phenotype than *WDR73* pathogenic variants. For these reasons, *OSGEP* variant analysis should be considered as the first step in genetic diagnosis of patients with clinical suspicion of GAMOS; this is especially true for those labs that do not perform massive parallel sequencing and for those cases with early and severe onset of the disease. Genetic diagnosis of GAMOS is of paramount importance for genetic counseling and family planning and allows prenatal or preimplantation genetic diagnosis for future pregnancies.

Abbreviations
CMRI: Cranial magnetic resonance imaging; DMS: Diffuse mesangial sclerosis; GAMOS: Galloway-Mowat syndrome; gnomAD: Genome Aggregation Database; KEOPS: Kinase, endopeptidase and other proteins of small size; TSE: Turbo Spin Echo

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
RT and EA designed and organized the study. DB, ABM, and EMM cared for the patients, acquired the clinical data, and prepared the samples from the family members. ADG, MF, MP, RT, and EA wrote the manuscript that was edited by all other authors. ADG, MP, and EA performed Sanger sequencing of the *OSGEP* gene. ADG, MP, and EA performed the NGS analysis, interpreted the NGS data, and drafted the genetic diagnostic report. RT and EA obtained funding. All authors read and approved the final manuscript.

Ethics approval and consent to participate
This study was approved by Fundació Puigvert Institutional Review Board. The parents of the patients provided written informed consent to participate in this study.

Consent for publication
The parents of the patients provided written informed consent to publish this case report, including case description, medical data, and images, maintaining anonymity.

Competing interests
RT is the Editorial Board Member of BMC Nephrology. The other authors declare that they have no competing interests.

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ARTICLE III: Clinical and genetic characterization of a cohort of proteinuric patients with biallelic *CUBN* variants

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SUMMARY

Background: Proteinuria is a well-known risk factor for progressive kidney impairment. Cubilin (*CUBN*) pathogenic variants have been related to Imerslund-Gräsbeck syndrome. Recently, C-terminal *CUBN* variants have been associated with isolated proteinuria without progression of kidney disease. In the era of genomic testing it is of crucial importance that independent case series provide genetic evidence for the assertion of new claimed gene-disease relationship.

Aims: The aims of this study were to perform clinical and genetic characterization of patients with proteinuria caused by anomalies in the *CUBN* gene.

Methods: Genetic testing of 347 families with proteinuria of suspected monogenic cause was performed by next-generation sequencing of a custom-designed kidney disease gene panel. Families with *CUBN* biallelic proteinuria-causing variants were studied at the clinical, genetic, laboratory, and pathologic levels.

Results: Twelve families (15 patients) bearing homozygous or compound heterozygous proteinuria-causing variants in the C-terminal *CUBN* gene were identified, representing 3.5% of the total cohort. We identified 14 different sequence variants, five of which were novel. The median age at diagnosis of proteinuria was 4 years (range 9 months to 44 years), and in most cases proteinuria was detected incidentally. Thirteen patients presented moderate-severe proteinuria without nephrotic syndrome. These patients showed lack of response to angiotensin-converting enzyme inhibitor (ACEi) and angiotensin receptor blocker (ARB) treatment, normal kidney biopsy, and preservation of normal kidney function over time. The two remaining patients presented a more severe phenotype, likely caused by associated comorbidities.

Conclusions: We confirm that the identification of C-terminal pathogenic *CUBN* variants is diagnostic of an entity characterized by glomerular proteinuria, normal kidney histology, and lack of response to ACEi/ARB treatment. This study increases awareness about albuminuria caused by C-terminal variants in the *CUBN* gene, which is a benign condition usually diagnosed

in childhood with preserved renal function until adulthood. In addition, our results will help to define *CUBN*-specific criteria for variant classification which could also be applied to other genes with a recessive mode of inheritance and causative of benign conditions with onset at paediatric age.

DISCUSSION

1. GENETIC TESTING IN EARLY-ONSET CKD

1.1. Diagnostic methodology: kidney disease gene panel

A precise diagnosis of patients with presumably monogenic causes of CKD based on clinical and/or histological phenotype and/or family history is generally complicated due to their high phenotypic overlap and huge genetic heterogeneity, with more than 400 genes causative or associated with IKD. Early detection of a monogenic cause for CKD can have important implications for patients and their family members, in terms of management, prognosis, genetic counseling and screening of family members. Genetic diagnosis can confirm a suspected clinical diagnosis, identify the molecular cause for CKD of unknown etiology, and distinguish among IKD with overlapping clinical manifestations. It is crucial for precise genetic counseling on family planning, guiding evaluation for manifestations in other organs, allowing for screening of at-risk family members, providing personalized treatment, and avoiding invasive diagnostic procedures such as renal biopsy.

With the advent of NGS, the possibilities of accurately diagnosing IKD have enormously increased. In recent studies, the overall diagnostic yield of genetic testing using NGS technology in patients with CKD was 30% in paediatric cohorts and 6-30% in adult cohorts (Groopman *et al.*, 2018; Lata *et al.*, 2018; Connaughton and Hildebrandt, 2019; Connaughton *et al.*, 2019; Ottlewski *et al.*, 2019). NGS technologies have enormously facilitated genetic diagnosis in recent times. It has dramatically increased the throughput and reduced the cost per nucleotide sequenced, enabling cost-effective sequencing of multiple genes in multiple patients. Many diagnostic laboratories use phenotype-associated targeted disease-specific gene panels as the first option in molecular diagnosis.

We previously developed a targeted sequencing of a kidney disease gene panel including 140 genes involved in cystic and glomerular IKD and demonstrated that it was a cost-and time-effective approach for genetic diagnosis of cystic and glomerular IKD (Bullich *et al.*, 2018). In terms of costs, it was estimated to save 40% of costs per sample compared to sequential Sanger sequencing and MLPA analysis of candidate genes. Given the heterogeneous disorders causative of IKD, the diagnostic success of disease-focused panels may be limited by difficulty in phenotyping kidney diseases into specific categories. Once this kidney disease gene panel was implemented in the diagnostic routine of patients with cystic and glomerular diseases, it was of interest to design a kidney disease gene panel capable to diagnose all monogenic IKD. In this thesis, a more extensive custom-designed kidney disease gene panel of 316 genes was developed to evaluate patients with different IKD, including cystic and glomerular diseases,

CAKUT, tubulopathies, and ADTKD (Figure 10) (Domingo-Gallego et al., 2021). This comprehensive approach that targets genes across a wide variety of kidney disease phenotypes allowed the detection of patients with phenocopies and was also useful in patients with CKD of unknown etiology, which could remain undiagnosed if a disease-focused panels were used.

Firstly, a comprehensive review of the literature was done on PubMed (https://www.ncbi.nlm.nih.gov/pubmed/) for genes causative of monogenic IKD. Genes were carefully selected from comparing different commercially available gene panels, and previously published literature research studies that used targeted gene panels or targeted gene panels exome-based (Nicolaou *et al.*, 2016; Heidet *et al.*, 2017; Groopman *et al.*, 2018; Van Der Ven *et al.*, 2018; Connaughton *et al.*, 2019). After a literature search process, we expanded our 140 gene panel (with a targeted region of 1.05 Mb, and mean depth of coverage 1393x) by adding genes causative of CAKUT, tubulopathies, ADTKD, and newly identified genes causative of cystic and glomerular diseases. In total, 316 genes causative of IKD were selected, with a targeted region of 2.02 MB and median depth of coverage of 635x. Secondly, the gene panel was designed using the NimbleGen SeqCap EZ Choice Library (NimbleGen; Roche). It was a refined design that included more probes to improve read depth over poorly covered regions, plus incorporate all exons and exon-intron boundaries (plus 20 base pairs at each end) of these 316 genes. Genome has some difficult-to-sequence regions that include genes that have pseudogenes or other highly homologous genomic regions, and longer stretches of repetitive sequences. For instance, *PKD1* is a very challenging gene due to its large size, complexity, and high GC content. The gene comprises 46 exons and the genomic region encompassing exons 1–33 shares 97.7% sequence homology with six pseudogenes on chromosome 16, making this region very difficult to resolve NGS and resulting in reduced sensitivity to detect disease-causing variants. The high level of DNA sequence identity with the pseudogenes creates the possibility for both false positive and negative genotype calls because a pseudogene variant can be incorrectly called as present in *PKD1*, and a *PKD1* variant can be easily missed. In addition, there is no mutational hot spot for *PKD1*, which means variants are usually private, highly variable and spread throughout the entire gene (Audrézet *et al.*, 2012). To be able to generate capture probes for the duplicated *PKD1* regions, we altered the settings for probe design of this specific region to allow probes to have up to 10 close matches in the genome. No probe redundancy was allowed in the final capture design for the rest of target regions. We validated its sensitivity, precision and specificity against several well-characterized controls. Finally, we applied our extensive custom-designed kidney disease gene panel for the

genetic diagnosis of 460 patients with suspected monogenic early-onset CKD (Domingo-Gallego *et al.*, 2021).

Some diagnostic laboratories prefer to use phenotype-associated gene panels that are WES-based. It is an attractive approach because it allows dynamic reanalysis of the data when new disease-causing genes are discovered. However, WES data has less sequence coverage than targeted gene panels. Our kidney disease gene panel achieved a complete coverage of all targeted regions at a high depth of coverage of the targeted genes, facilitating the detection of exon deletions and disease-causing variants in complex genomic regions of interest (such as homologous regions, repetitive regions, and GC-rich regions). It has also allowed the detection of disease-causing variants in a low proportion of reads in respect to the total number of reads, even in the *PKD1* gene, which in *de novo* cases is indicative of mosaicism. Our panel has also been able to detect some relative large deletion in which the capture probes detect the variant in a low proportion of reads but when validated by Sanger sequencing has been detected in heterozygous state. In addition, it has minimized the chance of incidental findings in genes unrelated to CKD.
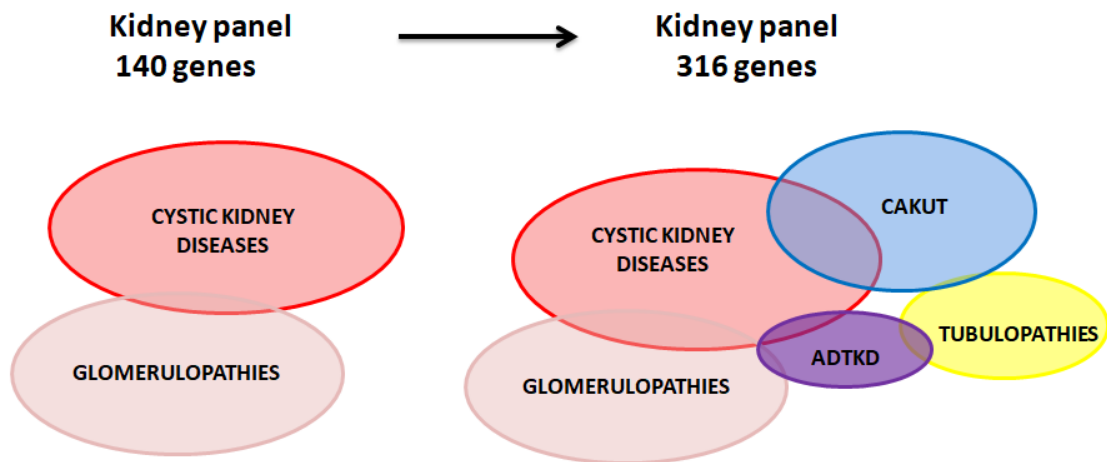
Figure 10. Schematic representation of the previous and actual version of our kidney disease gene panel. Abbreviations: ADTKD, autosomal dominant tubulointerstitial kidney disease; CAKUT, congenital anomalies of kidney and urinary tract.

1.2. Diagnostic rate of patients with suspected monogenic early-onset CKD

Our extensive kidney disease gene panel achieved a global diagnostic yield of 65% (300/460), which varied depending on the clinical diagnostic group: 77% in cystic kidney diseases, 76% in tubulopathies, 67% in ADTKD, 61% in glomerulopathies, and 38% in CAKUT (Figure 11).

Several studies have used NGS for genetic testing of patients with suspected IKD, achieving diagnostic rates ranging from 37% to 54% (Mallett *et al.*, 2017; Connaughton *et al.*, 2019;

Mansilla *et al.*, 2019; Jayasinghe *et al.*, 2021). Genetic testing of patients with no specific suspicion of monogenic CKD yielded lower diagnostic rates, 6–30% for adult (Groopman *et al.*, 2018; Ottlewski *et al.*, 2019; Yao *et al.*, 2019) and 30% for pediatric cohorts (Mann *et al.*, 2019).

Our high 65% diagnostic yield obtained in the present study must be interpreted in the light of certain considerations. First, our cohort included patients with CKD onset <30 years of age and was enriched by patients with a high suspicion of monogenic CKD, since 49% of them had a positive family history of kidney disease and 36% presented extrarenal manifestations. Second, our customized capture-based gene panel was optimized for efficient screening of difficult genomic regions, such as the duplicated region of the *PKD1* gene (Trujillano *et al.*, 2014). Genomic complexity of the *PKD1* gene may be the reason why several NGS studies have excluded patients with ADPKD from their cohorts (Mallett *et al.*, 2017; Lata *et al.*, 2018; Connaughton *et al.*, 2019; Mann *et al.*, 2019) or have reported a suboptimal coverage of *PKD1* (Groopman *et al.*, 2018). Third, we sequenced our custom gene panel at a high depth of coverage (median depth of 635×), allowing increased detection of exon deletions. Specifically, exon deletions were detected in 10% of all genetically diagnosed patients (31/300) in the following genes: *CLCNKB, COL4A5, HNF1B, IFT140, NPHP1, NPHP3, PKD1, SMARCAL1, TSC2*, and *TTC8*. Our high depth of coverage also allowed the detection of mosaic variants in two patients. Patient P45 and P171 with clinical diagnosis of ADPKD and TSC, were found to carry a mosaic disease-causing variant in 30% and 9% of the reads, respectively. These patients presented with a mild phenotype but they should be advised on the possibility of having severely affected offspring. To detect mosaic variants by NGS a high depth of coverage is required. Confirmation of these variants can be performed by Sanger sequencing if the proportion of mutated reads is approximately 10% or higher, and by allele-specific PCR o Snapshot in cases with less percentage of detected mutated reads. Finally, we complemented our genetic analysis with a specific method for the screening of the most frequent disease-causing variant in the *MUC1* gene. The predominant pathogenic variant in the *MUC1* gene [*MUC1* (NM_001204286.1): c.428dupC p.(Ala144Serfs*86)] causative of ADTKD is located in a tandem repeat region. This variant is not detectable by NGS or Sanger sequencing. The presence or absence of this cystosine duplication in patients with ADTKD can be assessed by SNaPshot minisequencing (Ekici *et al.*, 2014; Ayasreh *et al.*, 2018) or by mass spectrometry (Kirby *et al.*, 2013).
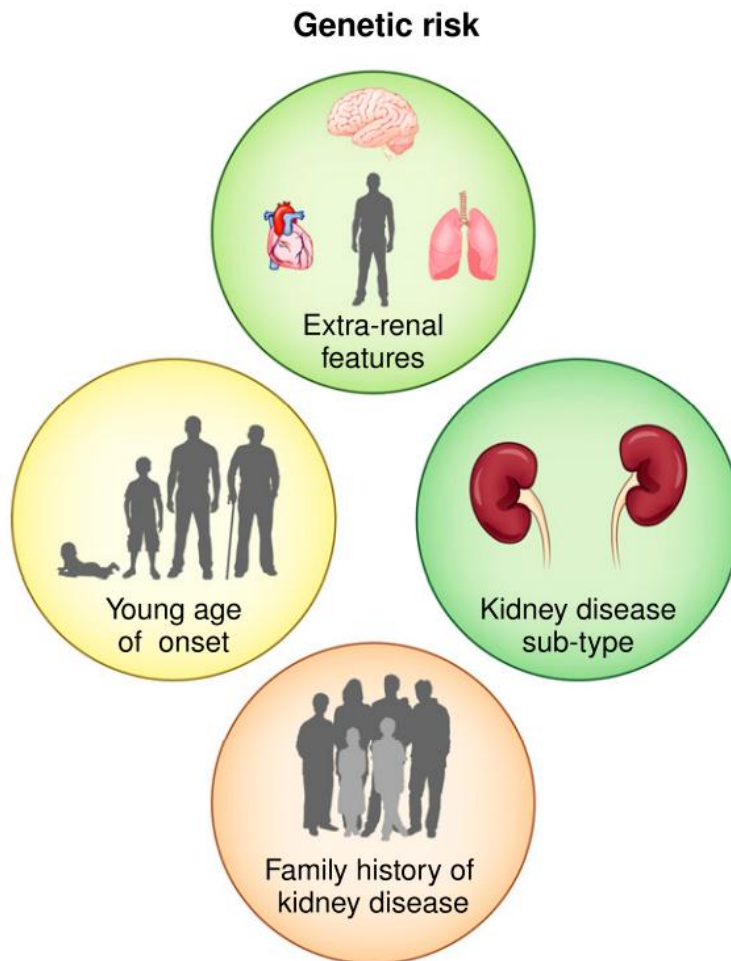
Figure 11. Clinical determinants of monogenic cause of CKD. Young age at onset, family history of kidney disease, and presence of extrarenal features are all predictive of genetic disease. Moreover, depending on the clinical diagnosis, the diagnostic yield of genetic testing varies. Adapted from (Cocchi and Nestor, 2020)**.**

Early onset of a disease should increase suspicion for a genetic disease. Hence, all children and young adults presenting with suspected genetic kidney disease or CKD of unknown etiology deserve genetic testing since age of onset significantly influence the probability that the cause is an underlying genetic diagnosis for certain clinical subtypes. However, it is not always accessible or affordable. In addition to young age at onset, family history of kidney disease, and presence of extrarenal features are all predictive of monogenic disease (Figure 11). Moreover, depending on the clinical diagnostic group, the diagnostic yield of genetic testing varies. Our strict inclusion criteria were intended to avoid genetic testing in patients in whom a monogenic cause was unlikely in order to maximize the cost-effectiveness of genetic testing. Our findings provide data to assist the prioritization of patients for genetic testing. We recommend performing genetic testing in all children diagnosed with cystic kidney disease or tubulopathy due to the high diagnostic yield identified in these disease groups. Among patients with CAKUT and glomerulopathy, we suggest that those with a family history of CKD

and/or extrarenal manifestations and for CAKUT those with bilateral alterations should be prioritized for genetic testing.

1.3. Most common causative genes of monogenic early-onset CKD

In total, disease-causing variants were identified in 64 of 316 genes included in our kidney-disease gene panel. Interestingly, just seven genes (*COL4A3, COL4A4, COL4A5, HNF1B, PKD1, PKD2,* and *PKHD1*) were responsible for 66% (198/300) of the diagnoses in our cohort of children and young adults (Figure 12.A). Similarly, a WES study of more than 3000 adult patients with CKD found that only six genes (*COL4A3, COL4A4, COL4A5, PKD1, PKD2,* and *UMOD*) accounted for 64% of the diagnoses in their adult cohort (Figure 12.B) (Groopman *et al.*, 2018). Causative variants were identified in 66 out of around 20,000 genes assessed by WES. While acknowledging outstanding differences between these two cohorts in terms of cohort size, inclusion criteria, the genetic approach used, and the diagnostic yield obtained, the two studies shared five genes (*COL4A3, COL4A4, COL4A5, PKD1,* and *PKD2*) among the most frequently mutated. The three genes found to be different in these studies can be explained by the different age at onset of CKD in the two cohorts: *HNF1B* and *PKHD1* are mainly causative of early-onset CKD and *UMOD* of adult-onset CKD. Considered in conjunction, the data from these two studies indicate that the identified eight genes may represent the main causative genes of monogenic CKD.
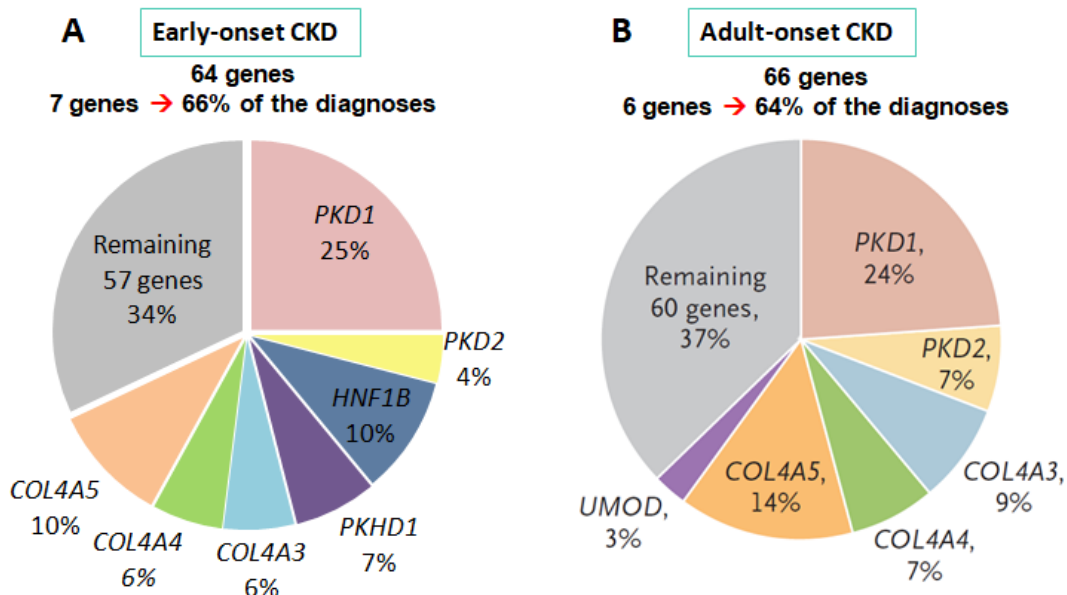

Figure 12. A. Most common causative genes identified in patients with early-onset CKD. B. Most common mutated genes identified in adult patients with CKD. Percentages do not total 100% because of rounding. Adapted from (Groopman *et al.*, 2018; Domingo-Gallego *et al.*, 2021)**.**

1.4. Clinical utility of genetic testing in patients with early-onset CKD

Among the 300 genetically diagnosed patients, the suspected clinical diagnosis was confirmed in 77%, a specific diagnosis within a clinical diagnostic group was identified in 15%, and 7% of cases were reclassified, which means that the genetic diagnosis identified a different cause than the clinically suspected. For patients with clinical suspicion of IKD, identification of the disease-causing variant(s) has several clinical implications as it provides a precise molecular diagnosis for the patients and their family members. An early genetic diagnosis may avoid the diagnostic odyssey that many patients face, with unnecessary and potentially harmful diagnostic procedures, multiple misdiagnoses and ineffective treatments. The main clinical implications of genetic testing are:

a) Confirm a suspected clinical diagnosis. For instance, identification of the causative pathogenic variants in the *PKHD1* gene in patient P142 confirmed his clinical diagnosis of ARPKD and allowed a precise reproductive genetic counseling for future offspring of their parents.

b) Reclassify a clinical diagnosis. For instance, molecular diagnosis different from the clinical diagnosis was found in fetus P124 with clinical suspicion of ARPKD due to enlarged kidneys, microcysts in collecting ducts, Potter facies and oligohydramnios. Genetic testing identified two disease-causing variants in *CPT2* gene allowing the diagnose of the severe infantile form of carnitine palmitoyl transferase II deficiency.

c) Identify molecular cause for kidney disease of unknown aetiology. For instance, patient P187 was diagnosed at birth with kidney-cysts of unknown aetiology. She had diabetes from infancy and reached KF at 23 years of age. The identification of biallelic pathogenic variants in the *PMM2* gene allowed for a precise diagnosis of polycystic kidney disease with hyperinsulinemic hypoglycemia (Cabezas *et al.*, 2017). To provide another example, patient P114 was an 8-month-old boy with prenatal detection of renal cysts with no specific clinical diagnosis. The detection of a frameshift variant in the *PKD1* gene gave rise to a precise diagnosis of ADPKD.

d) Enable precise genetic counselling that allows the patient and at-risk family members to be informed about the risk of transmitting the disease to his/her offspring and their relatives about their risk of suffering the disease. Also, it can guide decisions about family planning (prenatal or preimplantation genetic diagnosis) to couples in whom causative variant/s have been identified in a first affected child. In patient P77, identification of the causative variant in the *PKD1* gene permitted the patient to benefit from preimplantation genetic diagnosis.

e) Screen at-risk family members (presymptomatic testing), particularly useful in the setting of live related kidney donation and when there are available specific treatments, modification of life habits, genetic and reproductive counseling, among others.

f) Guide evaluation of extrarenal manifestations. For example, screening for diabetes and liver function in patients with a *HNF1B* disease-causing variant, for ocular anomalies in patients with *PAX2* disease-causing variant identified, and for hypoacusia and ocular anomalies in AS patients.

g) Avoid unnecessary diagnostic procedures such as kidney biopsy. For instance, in patients with AS confirmed by genetic diagnosis, as patient P229, an 18-year-old male with KF, hematuria, proteinuria, and bilateral high-tone sensorineural hearing loss, and possible lenticonus.

h) Guide choice of therapy. For example, two patients (P257 and P268) had causative variants in the *COQ8B* gene, which should trigger consideration of coenzyme Q10 supplementation (Montini, Malaventura and Salviati, 2008; Ashraf *et al.*, 2013). A genetic diagnosis can also prevent the prescription of ineffective therapies, such as immunosuppressive therapies in genetic forms of NS.

i) Provide prognosis information depending on which gene is mutated and the type of variant considering the genotype-phenotype correlations existing in some IKD such as ADPKD.

1.5. Variants of uncertain significance

The high throughput of NGS makes the size of a gene or its relative contribution to the disease no longer a limiting factor when deciding the content of a gene panel. However, the interpretation of the amount of data generated is challenging. The more genes included, the more variants to analyze and interpret. Our kidney disease-gene panel detects a ~6,000 variants per patient sample, meanwhile WES and WGS detect ~60,000 and ~300,000, respectively.

ACMG/AMP have developed a variant interpretation guidelines that has allowed harmonization of variant interpretation across laboratories and countries (Richards *et al.*, 2015). These guidelines incorporate different types of evidence (patient phenotype, population frequency, segregation and allelic evidence, computational and predictive data, functional assessment) at various levels of strength, and provide combining rules for a final classification in 5-tier classification system: benign, likely benign, uncertain significance, likely

pathogenic, pathogenic (Figure 9). The guidelines proposed strict use of the terms "likely pathogenic" and "likely benign", reserving it for variants with greater than a 90% certainty of being disease-causing or benign. One of the most challenging questions is how to deal with VUS, which is considered in the case of conflicting evidence, or when insufficient evidence is available to reach pathogenic or neutral significance of the variant. Data sharing initiatives are of particular importance in rare disease contexts. Variant databases with clinical information sharing initiatives, such as ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), HGMD (http://www.hgmd.cf.ac.uk/ac/search.php) and LOVD (https://www.lovd.nl/) databases, are imperative to establish clinically useful genotype–phenotype correlations and to maximize the benefit of genetic testing in routine nephrology practice. However, many missense variants identified in a patient have not been previously reported in any of these variant databases, and if they are absent or present in extremely low frequency in population databases and prediction algorithms predict to be pathogenic resulted classified as VUS. Thus, VUS account for a large amount of total identified variation. Additional analysis, such as family segregation and functional studies of the identified variant can provide new evidence on its pathogenicity. However, segregation analysis is not always possible, usually due to small family size and unwillingness of affected family members to participate in genetic testing, and functional assays are costly and time-consuming to develop. Thus, most of the variants remain classified as VUS.

It is unclear whether VUS should be included in the patient's report. Some laboratories report only pathogenic and likely pathogenic variants while others report VUS as well. Other laboratories report only hot VUS, i.e. those that even not accomplishing the criteria to be classified as likely pathogenic are located in a gene related to patient's phenotype, are absent in gnomAD and predicted to be pathogenic by several prediction algorithms. Another unsolved question is if these hot VUS have to be reported in the results section of the genetic report or in the annex. Often laboratories request if the patient wants VUS to be included in the report during the test ordering and informed consent process. Consequently, patients should be counseled both pre- and post-test about VUS in order to maximize their understanding, to ensure they have the capacity to make the decision, and minimize its negative emotional impact. Knowing that a VUS is present has limited clinical utility because it cannot be used for diagnosis or clinical decision-making, and sometimes can create confusion for patients and non-genetic health professionals. However, the possibility that new evidences about the pathogenicity of a VUS arise in the future allowing to upgrade a VUS to pathogenic or likely pathogenic status or to dismiss as benign, would have an immediate clinical impact both for

patients and their families. As reclassification of a VUS may occur years after the original test was performed, clinicians and patients may consider re-contacting the laboratory that performed the genetic testing periodically for updates. However, the classification of the pathogenicity of the identified variants and how to deal with VUS are still a major issue in genetic testing and there are still some unanswered questions. For instance, how often should VUS be reviewed for pathogenicity? What are the clinician's and laboratory's obligation for returning updated results to patients? But hopefully, collaboration and communication between clinicians, patients and laboratories, together with better guidelines and further research and application of machine learning techniques, will help to resolve these uncertainties.

1.6. Patients with suspected complex inherence pattern

Of the 300 patients with genetic cause identified, thirteen patients presented a putative complex inheritance pattern. We identified six patients with possible dual molecular diagnosis, three with a pathogenic variant and a likely hypomorphic variant in the *PKD1* gene, and four with suspected digenic AS involving two different *COL4A* genes.

Elucidating the contribution of additional variants in patients with putative complex inheritance patterns is difficult. Detailed phenotyping and segregation analysis is essential to interpret the pathogenicity of these combinations of variants (Rossetti *et al.*, 2009; Vujic *et al.*, 2010; Mencarelli *et al.*, 2015; Groopman *et al.*, 2018). However, it was not possible to obtain more detailed clinical phenotype or to segregate the identified variants in all thirteen patients. For that reason, we classify these patients as having a suspected complex inheritance pattern.

Oligogenic inheritance with changes in different genes have been previously reported in patients with CKD (Fliegauf, Benzing and Omran, 2007; Bergmann *et al.*, 2011; Groopman *et al.*, 2018).Six of these thirteen patients presented a dual molecular diagnosis defined by the identification of disease-causing variants in two different genes and ambiguous and complex phenotypes. Patient P101 presented enlarged kidneys, bilateral cysts, renal lithiasis and CKD stage III at 23 years of age. Molecular analysis revealed a frameshift variant in *PKD1* gene inherited from his mother affected of ADPKD. She also carried a likely hypomorphic variant in *PKD1* inherited from his asymptomatic father which may explain the earlier presentation in the proband. In addition, patient P101 carried a homozygous known causative variant of cystinuria. Patient P168 was prenatally diagnosed with Jeune syndrome and resulted in a legal termination of pregnancy. The fetus presented bilateral ureterohydronephrosis with cystic

dilation of some renal tubules in the necropsy, small thorax, pancreatic fibrosis, ductal plate malformation, shortened femurs and trident pelvis. As his brother affected with ARPKD, the fetus carried one homozygous disease-causing variant in the *PKHD1* gene. The fetus also carried a homozygous disease-causing variant in the *DYNC2H1* gene (causative of Jeune asphyxiating thoracic dystrophy, a NPHP-RC). Patient P204 was a 30 years old woman with clinical features and skin biopsy compatible with AS, CKD stage III and bilateral coloboma. She carried disease-causing variants in the *COL4A3* and *PAX2* genes and presented clinical manifestations of diseases caused by both genes. Patient P218 presented with microhematuria and proteinuria at 4 years of age. His renal biopsy showed minimal change disease by light microscopy, and diffuse effacement of the podocyte foot processes by electron microscopy. He carried two heterozygous variants in the *NPHS1* gene, and a heterozygous variant in the *COL4A4* gene. He shared the last variant with his father diagnosed with ADAS. They also shared one of the *NPHS2* variants, but it was not possible to confirm if the proband carried the *NPHS2* variants in *cis* or in *trans*. Patient P106 was a 2-years-old boy prenatally diagnosed with renal cysts. He presented multiple bilateral renal cysts and CKD stage II at 2 years of age. Several family members were diagnosed with ADPKD. We identified that the proband harbored a likely pathogenic variant in *PKD1* and *HNF1B* genes. Therefore, we consider that both detected variants may explain the early and severe presentation in the proband, but segregation analysis in family members could not be performed. Patient P3 was 2-years-old boy diagnosed with bilateral multicystic kidneys and left kidney hydronephrosis at 2-months of age. He was referred with a suspicion of *HNF1B*-related disease. He did not carry any disease-causing variant in the *HNF1B* gene or in other CAKUT causative genes. Instead, we identified two frameshift variants in the *COL4A3* and *PKD1* genes. The identified *PKD1* variant could be the cause of the renal cysts identified in the proband, but we cannot predict if the *COL4A4* variant will aggravate the phenotype with age.

The molecular genetic basis underlying complex inheritance of ADPKD have been elucidated with the discovery of hypomorphic or incompletely penetrant alleles as causative and modulators of the disease (Rossetti *et al.,* 2009). Co-inheritance of an inactivating *PKD1* variant *in trans* with a *PKD1* hypomorphic allele is associated with early-onset disease; harbouring homozygous or compound heterozygous hypomorphic alleles may cause typical ADPKD or a severe ARPKD-like disease; and carrying one hypomorphic allele in heterozygous state results in a mild cystic disease or may even be asymptomatic (Rossetti *et al.,* 2009; Vujic *et al.,* 2010). We identified three patients with cystic kidney disease that carried one pathogenic variant and a likely hypomorphic variant in the *PKD1* gene. Clinical evidence and segregation analysis could

be assessed in two of the patients (P55, P80). The remaining patient (P71) had an earlier presentation than a typical ADPKD, but had no available family members to analyze whether the variants were inherited *in cis*, *in trans*, or were *de novo*. As all three patients presented a more severe phenotype than the one expected by a disease-causing variant in the *PKD1* gene, we suggested a contribution of both alleles to the disease phenotype in the three of them.

Four patients with clinical diagnosis of AS presented a suspected digenic AS (P194, P205, P228 and P230). The patients harbored variants in two of the *COL4A* genes. However, in these patients, no more family members were available to assess the contribution of both variants to the phenotype. Opposite to the literature, these patients were not more severely affected (Furlano *et al.*, 2021).

1.7. Main limitations of our study

The main limitation of using a targeted kidney gene panel instead of WES is that recently identified genes causative of IKD have not been included (such as *DZIP1L* and *PARN*), which involves that periodic updating of the panel is necessary. Also, it is not possible to discover novel causative gens of monogenic CKD and has limited capacity for sequence reanalysis. In addition, in 25% (23/92) of patients with autosomal recessive disease it was not possible to confirm that the two identified variants were *in trans* since samples from parents were not available. Finally, our high genetic diagnostic rate is explained by the probable monogenic cause of the selected patients, and the rate is likely to be lower in unselected patients with early-onset CKD.

2. GALLOWAY- MOWAT SYNDROME: SEVERE EARLY-ONSET NS CAUSED BY VARIANTS IN THE *OSGEP* GENE

Galloway-Mowat syndrome (GAMOS) (OMIM #251300) is an extremely rare AR syndrome first described in 1968. It is a clinically heterogeneous condition characterized by early-onset NS associated with microcephaly, gyral abnormalities of the brain, and delayed psychomotor development. Most patients also present dysmorphic facial features, including hypertelorism, ear abnormalities, and micrognathia. Most affected individuals die in early childhood (Galloway and Mowat, 1968; Braun *et al.*, 2017). The estimated prevalence is <1/1,000,000 but it is likely that many cases remain misdiagnosed or undiagnosed.

In this thesis, we present the clinical and genetic characteristics of two unrelated infants with clinical suspicion of GAMOS who were born from consanguineous parents. Both patients

showed a similar clinical presentation, with early-onset NS, microcephaly, brain atrophy, developmental delay, axial hypotonia, and early fatality. At the moment of the referral, only disease-causing variants in the *WDR73* gene were known to cause GAMOS. We did not identify any disease-causing variants in this gene by Sanger sequencing.

Later, pathogenic variants in the *OSGEP, LAGE3, TP53RK*, and *TPRKB* genes were identified as novel monogenic causes of GAMOS (Braun *et al.*, 2017). These genes encode four subunits of the evolutionary highly conserved KEOPS (kinase, endopeptidase, and other proteins of small size) complex. We included these genes in the updated version of our costumed-designed kidney disease gene panel and sequenced both patients. We identified that the patients carried homozygous disease-causing variants in the newly identified *OSGEP* gene.

The *OSGEP* gene encodes the O-sialoglycoprotein endopeptidase enzyme, which regulates the second biosynthetic step in the formation of N-6-threonylcarbamoyladenosine in the cytosol, essential for mRNA translational initiation and efficiency. The highly conserved KEOPS complex is implicated in several cell processes, such as control of telomere length, telomere-associated DNA damage response signaling, and genome maintenance. Zebrafish larvae knockout of the osgep gene resulted in primary microcephaly, with increased apoptosis in the brain compared with controls and early lethality. Knockout mouse embryos also showed microcephaly compared with wild-type embryos. Neither mutant fish nor mice showed any renal phenotype, possibly due to embryonic early lethality (Braun *et al.*, 2017). Since that time, new genes have been reported to cause GAMOS (Braun *et al.*, 2018; Mann *et al.*, 2021). However, the genetic etiology of about three-quarters of patients with a clinical diagnosis of GAMOS remains elusive, suggesting that additional causative genes remain to be identified.

These two cases, in conjunction with the reported cases in the literature, add evidence that *OSGEP* disease-causing variants are the most prevalent cause of GAMOS and are associated with a more severe phenotype than *WDR73* disease-causing variants (Domingo-Gallego *et al.*, 2019). Making a genetic diagnosis available through publications or public genetic variant databases can offer confirmation and can inform and accelerate the diagnosis of future patients. Identification of the disease-causing variants in patients 1 and 2 confirmed the initial clinical suspicion of GAMOS and allowed precise genetic counseling to their parents. In particular, it allowed prenatal diagnosis of a baby girl without GAMOS for the parents of patient 1. In addition, these patients highlight the importance to offer genetic testing using an extensive updated gene panel.

3. BENIGN PROTEINURIA CAUSED BY VARIANTS IN THE *CUBN* GENE

Pathogenic variants in the *CUBN* gene were first described as causative of Imerslund-Gräsbeck syndrome (OMIM #261100), a rare autosomal recessive condition that is characterized by intestinal malabsorption of vitamin B_{12} resulting in megaloblastic anemia, frequently accompanied by varying degrees of proteinuria (Aminoff *et al.*, 1999; Birn and Christensen, 2006). Most pathogenic *CUBN* variants causative of IGS are found in the N-terminal half of cubilin, affecting either the interaction with amnionless or the vitamin B_{12}/intrinsic factor binding CUB domains 5–8 (CUB5–8). Ovunc *et al.* first described two siblings with isolated proteinuria in the absence of megaloblastic anemia carrying *CUBN* biallelic variants (Ovunc *et al.*, 2011). Later, a second family was reported with isolated proteinuria due to biallelic pathogenic *CUBN* variants (Jayasinghe *et al.*, 2019). Recently, C-terminal pathogenic *CUBN* variants have been found in patients with isolated proteinuria and normal kidney function. This finding contrasts with the general dogma that proteinuria is damaging and eventually causes kidney impairment (Bedin *et al.*, 2020).

In this thesis, we have confirmed that pathogenic C-terminal variants in the *CUBN* gene cause a benign proteinuric condition by reporting the genetic and clinical characterization of 15 patients of an independent cohort. In addition, this study should help to increase awareness among nephrologists of the good prognosis of these patients and to avoid unnecessary kidney biopsies and inefficient treatment for reduction of glomerular proteinuria. Better understanding of potential genetic causes of proteinuria should encourage genetic testing in patients with chronic proteinuria, especially in young patients with family history of proteinuria and/or consanguinity. Genetic testing enables the etiologic diagnosis of rare genetic causes of proteinuria in children and young adults such as Dent disease or juvenile cystinosis, commonly overlooked (Servais *et al.*, 2008; Frishberg *et al.*, 2009; Beara-lasic *et al.*, 2020), or glomerulopathies related to *LMX1B* and *PAX2,* for which clinical presentation may overlap (Boyer *et al.*, 2013; Barua *et al.*, 2014). All together support the value of genetic testing using massive parallel sequencing to achieve an accurate etiologic diagnosis of proteinuria and improve patient management.

When using the 2015 ACMG/AMP guidelines for variant classification in the *CUBN* gene, we found some difficulties. Three of the identified variants resulted classified as VUS. However, we considered these variants causative of chronic proteinuria with normal renal function for several reasons: i) they were identified *in trans* with another rare *CUBN* variant or in

homozygosis; ii) the phenotype of patients carrying these variants fitted with the one caused by the *CUBN* gene; iii) two of these variants were present in multiple of the identified patients.

One limitation of the 2015 ACMG/AMP guidelines is that were intended to be broadly applicable, but by nature of their generality, gene- and disease-specific questions must be individually determined. As previously mentioned, the ACMG/AMP guidelines proposed strict use of the term "likely pathogenic", reserving it for variants with greater than a 90% certainty of being disease causing. This narrow interval of pathogenicity is important for severe monogenic diseases for which identification of pathogenic and likely pathogenic variants may have therapeutic or reproductive consequences, but may not be applicable to variants causing of more benign conditions. Biallelic C-terminal *CUBN* variants cause chronic proteinuria with normal renal function, which is a benign condition that has no effect on reproductive fitness. This limitation was already recognize by the authors of the guidelines that wrote "that those working in specific disease groups should continue to develop more focused guidance regarding the classification of variants in specific genes given that the applicability and weight assigned to certain criteria may vary by gene and disease" (Richards *et al.*, 2015).

The National Institute of Health (NIH)-funded ClinGen consortium was formed in 2013 to develop standards and processes for evaluating genes and genomic variation to enhance clinical validity and utility. A key mission of ClinGen is to provide expert assessment of the clinical significance of genomic variants based on systematic and high-quality evidence review. Curation and expert review at this scale require a multi-institutional and interdisciplinary membership that ClinGen has organized through the development of subspecialty Variant Curation Expert Panels (VCEPs), each focused on a particular group of genes or diseases (Rivera-Muñoz *et al.*, 2018). As of this work, specifications for variant curation have been developed and published for a number of genes and diseases thus far (Gelb *et al.*, 2018; Kelly *et al.*, 2018; Lee *et al.*, 2018; Mester *et al.*, 2018; Oza *et al.*, 2018; Rivera-Muñoz *et al.*, 2018; Zastrow *et al.*, 2018; Luo *et al.*, 2019; Fortuno *et al.*, 2021) and other diseases and genes are in progress.

This thesis adds evidence that C-terminal *CUBN* variants cause chronic proteinuria with normal renal function. In addition, the characteristics of the *CUBN* variants identified would help to define *CUBN*-specific rules for variant classification which could also be applied to other genes with a recessive mode of inheritance and causative of benign conditions with onset at pediatric age. Further studies of patients with biallelic C-terminal *CUBN* variants and functional studies, together with expert specifications of the ACMG/AMP variant interpretation guidelines for

benign recessive conditions, will shed more light on the classification and causality of *CUBN* variants identified in the future.

CONCLUSION

1. The use of an extensive custom-designed kidney disease gene panel, sequenced at high-deep coverage, together with *MUC1* disease-causing variant analysis and specific inclusion criteria, provide a high diagnostic yield in patients with early-onset CKD of suspected monogenic cause.

2. Disease-causing variants in seven genes (*COL4A3, COL4A4, COL4A5, HNF1B, PKD1, PKD2*, and *PKHD1*) are the most common monogenic cause of early-onset CKD.

3. The likelihood of positive genetic diagnosis in early-onset CKD depends on the clinical diagnostic group, being highest for cystic kidney diseases and tubulopathies.

4. Genetic diagnosis is crucial in establishing a precise diagnosis in patients with early-onset CKD, especially for those with undiagnosed kidney disease. It also allows accurate genetic counselling and improves patient management.

5. *OSGEP* disease-causing variants are the most prevalent cause of GAMOS and are associated with a more severe phenotype than *WDR73* disease-causing variants.

6. Biallelic C-terminal variants in the *CUBN* gene cause a benign proteinuric condition characterized by isolated chronic proteinuria and normal kidney function.

7. The broad scope of ACMG guidelines necessitates specification of evidence types for specific genes or diseases. *CUBN*-specific criteria for variant classification may also be applied to other genes with a recessive mode of inheritance and causative of benign conditions with onset at paediatric age.

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