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MOLECULAR STUDY OF IDIOPATHIC NEPHROTIC SYNDROME

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STUDY III

Contribution of *TTC21B* gene to glomerular and cystic kidney diseases

SUMMARY

Background

Mutations in the *TTC21B* gene were initially reported as causative of ciliopathies. The homozygous p.P209L mutation was described in patients with nephronophthisis (NPHP) but it has recently been identified in families with FSGS and tubulointerstitial lesions. Heterozygous *TTC21B* variants have been suggested to interact *in trans* with other disease-causing genes and contribute as putative second-site modifiers in patients with cystic disease.

Aims

The aims of this study were to analyze the causative role of *TTC21B* gene mutations in our cohort of patients with a primary diagnosis of FSGS or NPHP as well as to assess the contribution of heterozygous *TTC21B* modifying alleles to glomerular and cystic kidney diseases.

Materials and methods

Mutation analysis of *TTC21B* gene was performed by massive parallel sequencing. To study the causative role of the *TTC21B* gene, we considered 17 patients with a primary diagnosis of SRNS/FSGS (n=15) or NPHP (n=2) and no mutations previously identified. To study its putative modifier role, we performed a case-control study considering 184 patients (368 alleles) with inherited glomerular or cystic kidney diseases and 2504 controls (5008 alleles).

Results

Disease-causing mutations in *TTC21B* were identified in three families presenting with nephrotic proteinuria and showing FSGS and tubulointerstitial lesions on renal biopsy. Some family members also presented hypertension and myopia. Two families carried the homozygous p.P209L and the third was compound heterozygote for the p.P209L and a novel p.H426D mutation. Heterozygous *TTC21B* variants predicted to be pathogenic were found in five patients. The frequency of these variants was significantly higher in renal patients compared to controls. However, when considering the total set of heterozygous *TTC21B* deleterious variants, a similar frequency was found in renal cases and controls. Two patients with a *TTC21B* variant, in addition to the disease-causing mutation, presented a more severe phenotype than expected.

Conclusions

The causal role of the homozygous p.P209L *TTC21B* mutation in patients with FSGS and tubulointerstitial disease is confirmed. In addition, a novel *TTC21B* mutation has been identified, demonstrating that p.P209L is not the only causative mutation of this disease. Thus, *TTC21B* mutation analysis should be considered for the genetic diagnosis of families with FSGS and tubulointerstitial lesions, who may also have hypertension and myopia. Finally, evidence is provided that heterozygous deleterious *TTC21B* variants may act as genetic modifiers of the severity of glomerular and cystic kidney diseases.

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Original Article

Contribution of the *TTC21B* gene to glomerular and cystic kidney diseases

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ABSTRACT

Background. The *TTC21B* gene was initially described as causative of nephronophthisis (NPHP). Recently, the homozygous *TTC21B* p.P209L mutation has been identified in families with focal segmental glomerulosclerosis (FSGS) and tubulointerstitial lesions. Heterozygous *TTC21B* variants have been proposed as genetic modifiers in ciliopathies. We aimed to study the causative and modifying role of the *TTC21B* gene in glomerular and cystic kidney diseases.

Methods. Mutation analysis of the *TTC21B* gene was performed by massive parallel sequencing. We studied the causative role of the *TTC21B* gene in 17 patients with primary diagnosis of FSGS or NPHP and its modifying role in 184 patients with inherited glomerular or cystic kidney diseases.

Results. Disease-causing *TTC21B* mutations were identified in three families presenting nephrotic proteinuria with FSGS and tubulointerstitial lesions in which some family members presented hypertension and myopia. Two families carried the homozygous p.P209L and the third was compound heterozygous for the p.P209L and a novel p.H426D mutation. Rare heterozygous *TTC21B* variants predicted to be pathogenic were found in five patients. These *TTC21B* variants were significantly more frequent in renal patients compared with controls ($P = 0.0349$). Two patients with a heterozygous deleterious *TTC21B* variant in addition to the disease-causing mutation presented a more severe phenotype than expected.

Conclusions. Our results confirm the causal role of the homozygous p.P209L *TTC21B* mutation in two new families with FSGS and tubulointerstitial disease. We identified a novel *TTC21B* mutation demonstrating that p.P209L is not the unique causative mutation of this nephropathy. Thus, *TTC21B* mutation analysis should be considered for the genetic diagnosis of families with FSGS and tubulointerstitial lesions. Finally, we provide evidence that heterozygous deleterious *TTC21B* variants may act as genetic modifiers of the severity of glomerular and cystic kidney diseases.

Keywords: FSGS, modifier, mutation, *TTC21B*, tubulointerstitial

INTRODUCTION

Identification of the *TTC21B* gene as causative of focal segmental glomerulosclerosis (FSGS) has broadened the phenotypic variability associated with this gene, initially described as causative of nephronophthisis (NPHP) [1, 2]. The *TTC21B* gene encodes the ciliary protein IFT139, a component of the intraflagellar transport complex A required for retrograde intraflagellar transport in the cilium [3].

Mutations in the *TTC21B* gene have been identified in nine ciliopathy patients: three with Jeune asphyxiating thoracic dystrophy (JATD), a chondrodysplasia that often leads to death in infancy [2, 4]; three with NPHP and extrarenal manifestations [2, 5] and three with isolated NPHP, all of whom carried the homozygous p.P209L mutation [1, 2]. The homozygous p.P209L mutation was identified in seven families with a primary diagnosis of FSGS [1]. Therefore, a total of 16 families with *TTC21B* mutations have been reported, 10 of which carried the homozygous p.P209L mutation. All of these 10 families were found to share the same haplotype, indicating a founder effect for this mutation in patients of Portuguese or North African origin. Re-examination of the renal biopsies of these patients revealed FSGS together with tubular lesions characteristic of NPHP disease. Their clinical features were late-onset proteinuria, high blood pressure and end-stage renal disease (ESRD) between 15 and 32 years. Functional studies on the effect of this mutation showed that it partially altered cilia structure, cell migration and cytoskeleton, suggesting a hypomorphic effect [1].

The phenotypic variability observed in glomerular and cystic hereditary kidney diseases supports the hypothesis that mutations in multiple genes encoding proteins that converge in common pathomechanistic pathways may influence clinical presentation and outcome. Heterozygous *TTC21B* variants have been suggested to interact *in trans* with other disease-causing genes and contribute as putative second-site modifiers in patients with cystic disease. Therefore, the *TTC21B* gene has been proposed to have both a causative and a modifying role in these patients [2]. To date, the possible effect of heterozygous *TTC21B* variants in glomerular diseases has not been studied.

Here, we aimed to study the causative role of *TTC21B* gene mutations in our cohort of patients with a primary diagnosis of FSGS or NPHP and no previously identified mutations, as well as to assess the contribution of heterozygous *TTC21B* modifying alleles to glomerular and cystic kidney diseases.

MATERIALS AND METHODS

Patients

A cohort of 187 patients with putative or molecularly confirmed inherited glomerular or cystic disease was analysed for *TTC21B* mutations. To study the causative role of the *TTC21B* gene, we considered 15 patients with steroid-resistant nephrotic syndrome (SRNS)/FSGS with likely genetic aetiology, in whom no mutations had previously been identified in 26 glomerular disease genes [6]. We also included two patients with NPHP disease in whom the homozygous *NPHP1* deletion had been ruled out. To study the putative modifying role of the *TTC21B* gene, we included the complete cohort, except for the three cases with *TTC21B* causative mutation. In most of these patients, a disease-causing mutation had been previously identified and the results have been partially published [6, 7]. These 184 patients had the following diagnosis: 48 autosomal dominant polycystic kidney disease (ADPKD), 24 autosomal recessive polycystic kidney disease, 6 *HNF1B* nephropathy, 6 tuberous sclerosis, 2 medullary cystic kidney disease, 1 NPHP, 47 Alport syndrome (AS) and 50 SRNS/FSGS. The study was approved by the Institutional Review Board and all participants gave their informed consent.

Sequencing and data analysis

All 29 *TTC21B* exons and intron boundaries (plus 100 bp at each end) were sequenced by targeted massive parallel sequencing. Libraries were prepared with the TruSeq DNA Sample Preparation Kit (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. Enrichment was performed with the custom NimbleGen SeqCap EZ Choice Library (Roche Nimblegen, Madison, WI, USA). Our design included probes to target additional genes beyond the scope of this study. Sequences were run on a HiSeq2000 platform (Illumina). Data analysis was performed with an in-house developed pipeline [8]. Briefly, all variants were required on both sequenced DNA strands and to account for ≥20% of total reads in that site. Polymorphisms present in ≥5% in dbSNP 138, the 1000 Genomes Project and the Exome Variant Server were discarded. Common benign variants and recurrent artefact variant calls were filtered out using our in-house exome variant database. All candidate pathogenic variants in the *TTC21B* gene were validated by conventional polymerase chain reaction amplification and Sanger sequencing.

Evaluation of the pathogenicity of the variants

The pathogenicity of the missense variants was evaluated considering their conservation among orthologs, population data and three *in silico* predictors (SIFT, Polyphen and Mutation Taster). Segregation of these variants with the disease was assessed for all available family members. The missense variants conserved among orthologues, with a minor allele frequency <0.01, and predicted to be deleterious by all the *in silico* predictors used, were considered to be pathogenic. Truncating mutations were considered definitely pathogenic.

Evaluation of the putative modifying role of *TTC21B* deleterious variants

To study the putative contribution of *TTC21B* deleterious alleles to cystic and glomerular diseases, a case–control association study was performed. The allelic frequencies of *TTC21B* pathogenic variants in our cohort of 184 patients (368 alleles) and 2504 controls (5008 alleles) were compared by means of the two-tailed Fisher's exact test. A P-value <0.05 was considered statistically significant. Allelic frequencies of control individuals were obtained from 1000 Genomes data phase III [9].

RESULTS

TTC21B causative mutations

We identified disease-causing mutations in the *TTC21B* gene in 3 of the 17 FSGS/NPHP cases (Table 1). Family 64 is a Spanish kindred with two siblings presenting with FSGS (Figure 1). Patient 64-1 presented with nephrotic proteinuria at the age of 4 years, and myopia. She did not respond to corticosteroid and immunosuppressive therapy and reached ESRD at the age of 6 years. Her renal biopsy showed FSGS with diffuse interstitial inflammatory infiltrate. Her brother (64-2) was diagnosed at the age of 6 years, due to his sister's disease, and presented with nephrotic proteinuria, chronic kidney disease (CKD) and high myopia. His biopsy findings showed FSGS with atrophic tubules and interstitial inflammatory infiltrate. He was not treated with corticosteroid or immunosuppressive therapy and he reached ESRD at the age of 8 years. Both siblings received a kidney transplant with no recurrence of the disease after 8 years of follow-up. They carried compound heterozygous *TTC21B* mutations: c.626C>T (p.P209L) and c.1276C>G (p.H426D) located in exons 6 and 11, respectively.

The p.P209L mutation has been reported to have a hypomorphic effect [1, 2]. The p.H426D variant has not been previously described in the literature. This variant is predicted to be pathogenic because it alters an amino acid conserved throughout evolution to *Caenorhabditis elegans*, and it is predicted to be deleterious to protein function by three publicly available programmes (SIFT, Polyphen and Mutation Taster). Population data show that this variant is absent in 280 control chromosomes and the 1000 Genomes database, whereas it has been reported once (1/119278) in the ExAC database (Table 2).

Family 22 is a Moroccan kindred with two affected siblings. The only available family member was a 12-year-old girl diagnosed with nephrotic proteinuria and CKD at 12 years of age. She came to our hospital at the age of 14 years due to a hypertensive crisis presenting with ESRD. Her renal biopsy showed only three glomeruli, all with global sclerosis, tubulointerstitial fibrosis and atrophic tubules. She received a kidney transplant, with no recurrence of the disease after 2 years. She carried the homozygous p.P209L mutation. The only information from her brother was that he reached ESRD at 23 years of age.

Family 374 is a Moroccan kindred with five children, two of them affected. Patient 374-1 was diagnosed due to a hypertensive crisis at 8 years old. He presented with nephrotic range proteinuria, CKD stage V and high myopia, and his renal biopsy showed FSGS with interstitial fibrosis and tubular atrophy. He was resistant to steroid and immunosuppressive therapy and reached ESRD 2 months after diagnosis. Patient 374-2 has recently been diagnosed at the age of 17 years with nephrotic proteinuria and myopia. Her biopsy showed FSGS, small foci of tubulointerstitial fibrosis and atrophic tubules. Both siblings were homozygous for the p.P209L mutation.

Contribution of *TTC21B*-modifying alleles to glomerular and cystic diseases

We identified rare heterozygous *TTC21B* variants predicted to be pathogenic (Table 2) in 5 of the 184 patients: 4 with glomerular disease and 1 with cystic disease (Table 3). These *TTC21B* variants were significantly more frequent in renal patients compared with controls [cases = 1.36% (5/368) versus controls = 0.44% (22/5008); P = 0.0349]. However, a similar frequency for the total set of rare *TTC21B* variants predicted to be pathogenic was found between renal patients and controls [cases = 1.36% (5/368) versus controls = 1.68% (84/5008); P = 0.8324].

Two of the five patients with a *TTC21B* variant, in addition to the disease-causing mutation, presented a more severe phenotype than expected. Patient 61 with ADPKD presented with enlarged polycystic kidneys, high blood pressure and CKD stage IV at the age of 36 years. He carried a *de novo* missense mutation in the *PKD1* gene and additionally the p.L1002V *TTC21B* variant. He did not bear other candidate pathogenic variants in *PKD1*/*PKD2*/*PKHD1*/*HNF1B* genes

Table 1. Clinical and genetic data of patients with disease-causing mutations in the *TTC21B* gene

Family	Patient	Origin	Sex	Age at diagnosis (years)	Features at diagnosis	Other features	Glomerular lesions	Tubular lesions	Therapy and response	Age at ESRD (years)	Gene	Mutation 1	Mutation 2
64	64-1	Spain	F	4	Nephrotic proteinuria	Myopia	FSGS	Interstitial inflammatory infiltrate	Cs, CP–	6	*TTC21B*	c.626C>T p.(P209L)	c.1276C>G p.(H426D)
	64-2		M	6	Nephrotic proteinuria CKD	Myopia	FSGS	Interstitial inflammatory infiltrate, atrophic tubules	–	8	*TTC21B*	c.626C>T p.(P209L)	c.1276C>G p.(H426D)
22	22	Morocco	F	12	Nephrotic proteinuria CKD	HBP	Global sclerosis	Tubulointerstitial fibrosis, atrophic tubules	–	14	*TTC21B*	c.626C>T p.(P209L)	c.626C>T p.(P209L)
374	374-1	Morocco	M	8	Nephrotic proteinuria CKD	HBP, myopia	FSGS	Interstitial fibrosis, atrophic tubules	Cs, CP–	8	*TTC21B*	c.626C>T p.(P209L)	c.626C>T p.(P209L)
	374-2		F	17	Nephrotic proteinuria	Myopia	FSGS	Interstitial fibrosis, atrophic tubules	–	–	*TTC21B*	c.626C>T p.(P209L)	c.626C>T p.(P209L)

Therapy response: –, no response. Mutations on these genes were classified according to GenBank accession numbers: NM_024753.4 and NP_079029.3. The nomenclature used in this study for the description of sequence variants in DNA and protein is in accordance with the Human Genome Variation Society guidelines and can be found at http://www.hgvs.org/.
CKD, chronic kidney disease; CP, cyclophosphamide; Cs, corticosteroids; ESRD, end-stage renal disease; F, female; FSGS, focal segmental glomerulosclerosis; HBP, high blood pressure; M, male.

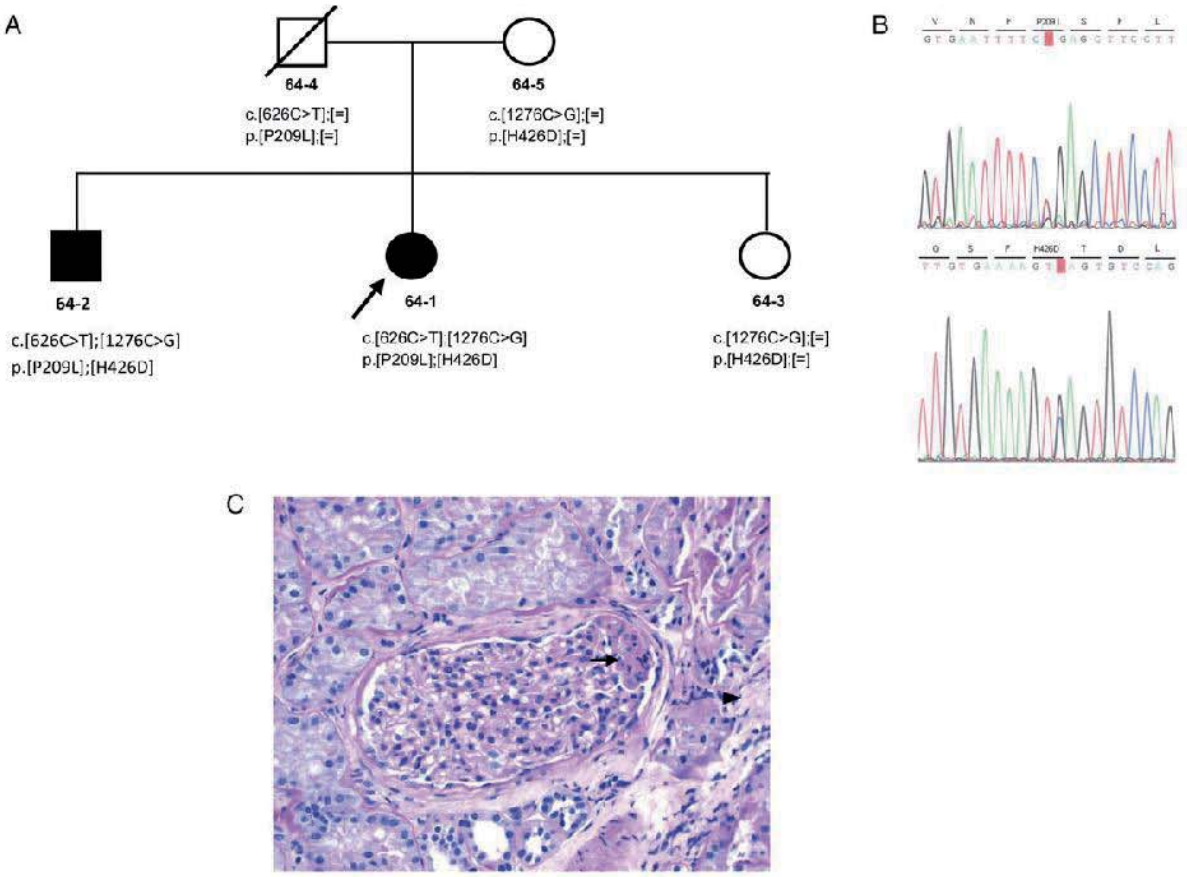

FIGURE 1: Mutations in the *TTC21B* gene in a kindred with familial FSGS. (**A**) Pedigree of family 64. The two affected siblings have compound heterozygous mutations in the *TTC21B* gene while the rest of the family members are healthy carriers of only one mutation. The arrow indicates the proband. Squares denote males, circles denote females. Filled symbols indicate affected status. Slashed symbol indicates a deceased individual. (**B**) Electropherogram of the c.626C>T (p.P209L) and c.1276C>G (p.H426D) heterozygous *TTC21B* mutations. (**C**) Representative kidney biopsy from patient 64-2 (a haematoxylin and eosin staining, magnification ×20) showing a focus of glomerular collapse with an increase in mesangial matrix (arrow), atrophic tubules and interstitial fibrosis (arrowhead).

Table 2. Potential impact of the amino acid variants found in the *TTC21B* gene

TTC21B variant[a]	Amino acid sequence conservation[b]	MAF in EVS	PolyPhen		SIFT		Mutation Taster		Functional study prediction
			Score	Prediction	Score	Prediciton	Score	Prediction	
P209L	*C. elegans*	0.0002	1.000	D	0.97	D	1.000	D	Hypomorphic[c,d]
T231S	*Gallus gallus*	0.0012	0.626	P	0.92	D	0.949	D	Hypomorphic[c]
H426D	*Xenopus tropicalis*	Not reported	1.000	D	0.96	D	0.994	D	NP
V559G	*Danio rerio*	0.0015	0.992	D	1.00	D	0.835	D	NP
R991H	*X. tropicalis*	0.0002	1.000	D	0.98	D	1.000	D	NP
L1002V	*C. elegans*	0.0066	0.958	D	1.00	D	0.999	D	Hypomorphic[c]

D, deleterious; EVS, exome variant server; MAF, minor allele frequency; P, possibly damaging; NP, not performed.
[a]*TTC21B* variants are numbered according to GenBank accession number: NP_079029.3.
[b]The amino acid residue is continually conserved throughout evolution as indicated.
[c]Performed by Davis *et al.* [2].
[d]Studied by Huynh Cong *et al.* [1].

[10]. Patient 82 presented with microhaematuria, full-blown nephrotic syndrome and high blood pressure at 28 years of age. Her renal biopsy showed FSGS with tubular atrophy and interstitial fibrosis and she reached ESRD at 32 years of age. She carried a *COL4A3* missense mutation in the heterozygous state together with a frameshift mutation in *TTC21B*. She had no other candidate pathogenic variants in the 26 glomerular gene panel [6].

The remaining three patients carrying an additional *TTC21B* variant had childhood-onset recessive diseases with short-term follow-up. Patient 186 was a 9-year-old girl with haematuria (onset at 11 months), non-nephrotic proteinuria (onset at 3 years), hearing loss (onset at 7 years) and hyperuricemia with normal renal function. Genetic diagnosis confirmed autosomal recessive AS caused by compound heterozygous mutations in

Table 3. Patients with glomerular or cystic disease and additional heterozygous pathogenic *TTC21B* variants

Patient	Origin	Sex	Clinical diagnosis	Type of renal disease	Disease causing			*TTC21B* variant	*TTC21B* allele frequencies	
					Gene	Mutation 1	Mutation 2		Patients (%)	Controls (%)[a]
61	Spain	M	ADPKD	Cystic	*PKD1*	c.9889G>A p.(V3297M)		c.3004C>G p.(L1002V)[b]	1/368 (0.27)	6/5008 (0.12)
82	Spain	F	ADAS	Glomerular	*COL4A3*	c.3044G>A p.(G1015E)		c.1713_1714delGT p.(Q571Hfs*13)	1/368 (0.27)	0/5008 (0.00)
156	Spain	M	XLAS	Glomerular	*COL4A5*	c.3605_3790del p.(G1202_P1263)		c.2972G>A p.(R991H)	1/368 (0.27)	1/5008 (0.02)
186	Spain	F	ARAS	Glomerular	*COL4A3*	c.4441C>T p.(R1481*)	c.40_63del p.(L14_L21del)	c.691A>T p.(T231S)[b]	1/368 (0.27)	7/5008 (0.14)
228[c]	African	F	SRNS	Glomerular	*NPHS2*	c.779T>A p.(V260E)	c.779T>A p.(V260E)	c.1676T>G p.(V559G)	1/368 (0.27)	8/5008 (0.16)

Mutations on these genes were classified according to GenBank accession numbers: NM_001009944.2 and NP_001009944.2 (*PKD1*); NM_000091.4 and NP_000082.2 (*COL4A3*); NM_000495.4 and NP_000486.1 (*COL4A5*); NM_014625.3 and NP_055440.1 (*NPHS2*); NM_024753.4 and NP_079029.3 (*TTC21B*). The nomenclature used in this study for the description of sequence variants in DNA and protein is in accordance with the Human Genome Variation Society guidelines and can be found at http://www.hgvs.org/.
ADAS, autosomal dominant Alport syndrome; ADPKD, autosomal dominant polycystic kidney disease; ARAS, autosomal recessive Alport syndrome; F, female; M, male; SRNS, steroid-resistant nephrotic syndrome; XLAS, X-linked Alport syndrome.
[a]Data from 1000 Genomes Project Phase 3.
[b]Mutations with functional studies (Davis *et al.* [2]).
[c]Only child of consanguineous parents.

COL4A3. She also carried the p.T231S *TTC21B* variant. Patient 228 was an only child of consanguineous parents diagnosed with nephrotic proteinuria at 8 years of age. Her renal biopsy showed FSGS. She did not respond to corticosteroid and immunosuppressive therapy and presented a rapid deterioration in renal function, reaching ESRD at 9 years of age. She carried a homozygous *NPHS2* mutation and additionally the p.V559G *TTC21B* variant. The DNA sample from Patient 156, a 6-year-old boy, arrived at our centre for genetic diagnosis of X-linked AS. A *de novo* mutation in *COL4A5* was identified. He also carried the p.R991H *TTC21B* variant. No clinical information was available for this patient.

DISCUSSION

In this study, we confirmed the causative role of *TTC21B* recessive mutations in patients with both FSGS and tubulointerstitial lesions and found that this phenotype is not caused by only homozygous p.P209L. Our findings support a putative modifying effect of rare *TTC21B* variants on glomerular and cystic disease severity based on the clinical manifestations of patients carrying heterozygous *TTC21B* variants in addition to the causative mutation.

Genotype–phenotype correlations in patients carrying recessive *TTC21B* mutations have been established. Patients with at least one truncating or splice site mutation exhibit JATD or early onset NPHP with extrarenal involvement [2, 4], whereas all patients carrying the homozygous p.P209L have isolated renal disease and display both FSGS and tubulointerstitial lesions on renal biopsy [1] (cf. families 22 and 374 described here). Moreover, our family 64 demonstrates that missense mutations different from the homozygous p.P209L can also cause this phenotype. A total of 23 patients with the FSGS and tubulointerstitial lesions due to homozygous or compound heterozygous missense mutations in the *TTC21B* gene have been described: 18 reported patients (10 families) [1] plus the 5 patients (3 families) of this study. Clinically, these patients presented with childhood or adult onset of proteinuria (in the nephrotic or non-nephrotic range) and a rapid progression to ESRD. Interestingly, the two affected siblings carrying the p.P209L and p.H426D mutation in the *TTC21B* gene had an younger age at onset of proteinuria (4 and 6 years) than the homozygous p.P209L patients (8–30 years, median 16 years). High blood pressure was present in 17 of the 23 cases (74%), indicating that it is a frequent clinical feature in patients with *TTC21B* mutations. Myopia was reported in 5 of 23 patients (22%). Given the rapid progression of the renal disease in these patients, it is difficult to determine whether the tubuloglomerular involvement is the primary lesion or the consequence of this rapid deterioration in renal function, but the focal and segmental aspect of the glomerular lesion and the interstitial infiltrate point to a specific pathologic setting for this disease.

Functional studies in zebrafish and mammalian cells have demonstrated that the p.P209L mutation is a hypomorphic allele because it allows partial function of the protein [2]. In human mature podocytes, the p.P209L mutation has been demonstrated to cause a partial alteration of the microtubule network that may affect cytoskeleton dynamics and destabilize podocyte architecture [1], thus supporting the hypothesis of its hypomorphic effect. To the best of our knowledge, the p.H426D mutation has not been previously reported in the literature, but its complete conservation throughout the evolution, the non-conservative nature of the substitution and the predicted deleterious effect by three different *in silico* tools strongly support its pathogenicity. Based on the earlier onset of proteinuria in the siblings carrying p.H426D, this variant might be more deleterious than the p.P209L mutation, although an effect of other modifying factors cannot be excluded.

TTC21B mutations were described to have a dual role, as causative of ciliopathies and second-site modifiers in patients with cystic diseases. This modifying role of heterozygous *TTC21B* variants was suggested by Davis *et al.* [2] based on their significant enrichment in ciliopathy patients. In our cohort, we did not find significant enrichment for the total set

of rare *TTC21B* pathogenic variants. However, when considering the five variants identified in patients, a significant association of the *TTC21B* gene with glomerular and cystic kidney diseases was found. The fact that, in our case–control study, we did not observe significant enrichment for the total set of rare *TTC21B* pathogenic variants might be explained by several differences between the two studies: (i) Davis *et al.* studied NPHP-related ciliopathies, while our cohort included mostly polycystic kidney diseases (PKDs) and glomerular diseases; (ii) the smaller size of our case cohort and (iii) Davis *et al.* sequenced their control group, while we obtained control data from 1000 Genomes.

Mutations in multiple renal disease genes may explain some of the phenotypic variability in glomerular and cystic hereditary kidney diseases. Bergmann *et al.* [10] described severely affected patients with PKD who carried, besides their familial mutation, additional alterations in PKD genes, which likely aggravate their phenotype. We recently described an increased disease severity in patients carrying mutations in an SRNS gene and also in the *COL4A3* gene [6]. Here, we describe two patients with a *TTC21B* variant, in addition to a heterozygous disease-causing mutation, who presented a more severe phenotype than expected. Patient 61 with ADPKD, carrying a missense *PKD1* mutation and a *TTC21B* heterozygous variant, presented with CKD stage IV at 36 years, whereas patients with *PKD1* non-truncating mutations have a median age at ESRD of 68 years [11]. Patient 82 with autosomal dominant Alport syndrome (ADAS) reached ESRD at 32 years, while only ∼29% of ADAS patients progress to ESRD at a mean age of 56 years [12]. Additional studies are needed to confirm the contribution of deleterious *TTC21B* alleles to the severity of glomerular and cystic diseases.

In conclusion, *TTC21B* mutation analysis should be considered for the genetic diagnosis of patients with FSGS and tubulointerstitial lesions who may also have hypertension and myopia, especially in familial cases. Our results suggest that deleterious *TTC21B* alleles likely aggravate the phenotype of patients with glomerular and cystic inherited diseases, adding support to the proposed modifier role of *TTC21B* alleles.

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CONFLICT OF INTEREST STATEMENT

None declared.

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STUDY IV

Clinical utility of a kidney-disease gene panel for genetic diagnosis of cystic and glomerular inherited kidney diseases

SUMMARY

Background

Molecular diagnosis of cystic and glomerular inherited kidney diseases (IKD) by Sanger sequencing is complicated because of the high genetic heterogeneity and phenotypic variability of these diseases. Cystic IKD mainly comprise autosomal dominant polycystic kidney disease (ADPKD), caused by mutations in the *PKD1* or *PKD2* genes, autosomal recessive polycystic kidney disease (ARPKD), due to *PKHD1* mutations and nephronophthisis-related ciliopathies (NPHP-RC) with more than 70 associated genes. Clinical manifestations of these diseases can overlap and also be mimicked by mutations in other genes. Glomerular diseases mainly include Alport syndrome (AS), caused by mutations in the *COL4A3*, *COL4A4* or *COL4A5* genes and SRNS with more than 25 causative genes identified. A more comprehensive approach for genetic diagnosis of cystic and glomerular IKD is required.

Aims

In this study, we aimed to develop a kidney-disease gene panel approach for 132 genes involved in cystic or glomerular IKD.

Materials and methods

Our kidney-disease gene panel was sequenced in 220 patients, a pilot study of 116 patients previously characterized and a diagnostic cohort of 104 patients.

Results

Targeted sequencing of our kidney-disease gene panel identified mutations in 115 of the 116 patients in the pilot study. A total of 134 mutations were detected including single nucleotide variants, small insertions or deletions and large structural variants, for a final sensitivity of 99%. Disease-causing mutations were identified in 75% of the diagnostic patients, including 90% of ADPKD patients, 88% of ARPKD, 78% of the tuberous sclerosis patients, 43% of cystic patients with indeterminate clinical diagnosis, 82% of AS patients, 40% of SRNS patients, and 100% of patients with hereditary angiopathy with nephropathy, aneurysms, and muscle cramps (HANAC). Our kidney-disease gene panel approach allows a more comprehensive analysis of patients with cystic and glomerular IKD by identifying 1) mutations in low-frequency mutated genes that may be differential diagnosis of IKD, 2) mosaic mutations, 3) structural variants, and 4) complex inheritance patterns.

Conclusions

Massive parallel sequencing of our kidney-disease gene panel is a comprehensive, efficient and cost-effective tool for genetic diagnosis of cystic and glomerular IKD. Our approach improves the genetic diagnosis by detecting all types of genetic variation, including structural variants and mosaic mutations, as well as by elucidating complex inheritance patterns.

KIDNEY-DISEASE GENE PANEL FOR A COMPREHENSIVE GENETIC DIAGNOSIS OF CYSTIC AND GLOMERULAR INHERITED KIDNEY DISEASES

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ABSTRACT

Introduction: Molecular diagnosis of cystic and glomerular inherited kidney diseases (IKD) is complicated by their high genetic heterogeneity and phenotypic variability. Clinical manifestations of these diseases cover a broad range of phenotypes that can be mimicked by mutations in several genes. We aimed to develop a more comprehensive approach for genetic diagnosis of these IKD.

Materials and methods: Massive parallel sequencing of 132 genes causative or associated with cystic or glomerular IKD was performed in 220 patients, a pilot study of 116 patients and a diagnostic cohort of 104 patients.

Results: We identified 134 of the 135 mutations (99%) in the validation cohort demonstrating similar sensitivity than Sanger sequencing. Disease-causing mutations were identified in 75% of the diagnostic patients, including 90% of ADPKD patients, 88% of ARPKD patients, 78% of tuberous sclerosis patients, 43% of cystic patients with inconclusive clinical diagnosis, 82% of AS patients, 40% of SRNS patients, and 100% of HANAC patients. Our kidney-disease gene panel approach allows a more comprehensive analysis by identifying 1) mutations in low-frequency mutated genes that may be differential diagnosis of IKD, 2) mosaic mutations, 3) structural variants, and 4) complex inheritance patterns.

Conclusions: Targeted sequencing of our kidney-disease gene panel is a comprehensive, efficient and cost-effective tool for genetic diagnosis of cystic and glomerular IKD.

INTRODUCTION

Inherited kidney diseases (IKD) are the leading cause of chronic kidney disease (CKD) in children and account for at least 10% of patients with ESRD in Europe [1]. Molecular diagnosis in IKD can help to provide a definitive diagnosis, to make treatment decisions, to provide prognostic information regarding the gene and type of mutations, to encourage living donor kidney transplantation, and to enable genetic counseling.

Cystic IKD are characterized by the formation of renal cysts that disrupt the structure of the nephron. Autosomal Dominant Polycystic kidney disease (ADPKD) is the most common IKD. It is typically a late-onset disease caused by mutations in *PKD1* or *PKD2.* Autosomal recessive polycystic kidney disease (ARPKD) generally presents in the perinatal period and is caused by mutations in the *PKHD1* gene. About 2-5% of ADPKD patients show an early and severe phenotype that is often clinically indistinguishable from ARPKD [2]. Some of these severely affected patients carry more than one mutation in polycystic genes, probably aggravating the phenotype [3]. Clinical manifestations of polycystic kidney disease can also be mimicked by mutations in *HNF1B* or genes that typically cause nephronophthisis-related ciliopathies (NPHP-RC), especially in the prenatal setting and early childhood [2]. NPHP-RC include a broad range of usually autosomal recessive (AR) diseases with childhood onset. More than 70 genes have been associated with NPHP-RC [4] of whom 18 are the most frequently mutated accounting for up to 21% of patients [5]. Autosomal dominant tubulointerstitial kidney disease (ADTKD) is a late-onset autosomal dominant disease (AD) with clinical and histological features similar to NPHP-RC. It is caused by mutations in *UMOD, MUC1*, *REN* or *HNF1B* genes [6]. Renal cysts are also a frequent manifestation of tuberous sclerosis (TS), caused by mutations in the *TSC1* or *TSC2* genes.

Glomerular IKD are caused by structural defects in the glomerular filtration barrier composed of three layers: the fenestrated endothelium, the glomerular basement membrane and the podocytes. Alport syndrome (AS) is caused by alterations in the collagen IV proteins of the glomerular basement membrane. Mutations in *COL4A5* gene are responsible for the X-linked form of the disease whereas mutations in *COL4A3* and *COL4A4* genes can be inherited following an AR or AD pattern, also named collagen type IV (α3α4) nephropathy. Steroid-resistant nephrotic syndrome (SRNS) and focal segmental glomerulosclerosis (FSGS) have typically been associated with podocyte mutations with more than 25 causative genes identified [7]. Heterozygous mutations in *COL4A3* or *COL4A4* can also exhibit FSGS on light

microscopy. In addition, patients carrying mutations in a SRNS gene and *COL4A3* presented a more severe phenotype [8].

The high genetic heterogeneity and phenotypic variability of most IKD makes genetic testing by Sanger sequencing expensive and time-consuming. In addition, all IKD, except for ADPKD, are rare diseases (that affect less than 5 persons in every 10,000) with low prevalence. Based on this scenario, an integrative approach for cost- and time-effective genetic diagnosis of cystic and glomerular IKD is required. Here, we developed a kidney-disease gene panel for genetic diagnosis of cystic and glomerular IKD.

MATERIALS AND METHODS

Patients

A total of 220 patients with IKD were considered, including a pilot study of 116 patients with previously known mutations and 104 diagnostic patients. The pilot-study patients carried different types of mutations in the following genes: *PKD1*, *PKD2*, *PKHD1*, *HNF1B, NPHP1*, *UMOD*, *TSC2*, *COL4A5*, *COL4A3*, *NPHS1*, *NPHS2*, *WT1*, *INF2*, *TRPC6*, and *GLA*. The diagnostic patients were referred to our laboratory for cystic or glomerular IKD genetic testing from January 2015 to January 2016 and consisted of 61 cystic IKD patients, 14 patients with cystic IKD phenotype but indeterminate clinical diagnosis, and 29 glomerular IKD patients. The study was approved by the Institutional Review Board, and all patients gave their signed informed consent.

Kidney-disease gene panel

We designed a kidney-disease gene panel including 132 genes causative of or associated with IKD in humans or animal models. Our kidney-disease gene panel comprises the genes associated with ADPKD, ARPKD, NPHP-RC, ADTKD, TS, AS, SRNS/FSGS, HANAC, and FABRY as well as genes that may be a differential diagnosis of these diseases (Supplementary Table 1). All exons and intron boundaries (plus 20bp at each end) of these genes were captured using a custom NimbleGen SeqCap EZ Choice Library (Roche NimbleGen; Madison, WI, USA). After removal of repetitive sequences, 99% of the targeted bases were covered with probes for a final targeted region of 1.05 Mb.

Library preparation and sequencing

Genomic DNA was isolated from peripheral blood using salting-out method. In familial cases, only the proband was analyzed by massive parallel sequencing. Libraries were prepared

according to manufacturer's standard protocol: NimbleGen SeqCap EZ Library SR version 4.3 and sequenced on a HiSeq2500 instrument. Data analysis was performed using open source in-house pipeline. Briefly, sequencing reads were aligned with BWA to the hg19 human reference genome and realigned with Indelrealigner. Variant calling was performed independently by 3 SNP callers (UnifiedGenotyper, HaplotypeCaller and Mpileup). All the variants called by at least one SNP caller were annotated with Annovar. Analysis of structural variants was performed using the CONTRA tool [9]. A schematic representation of the workflow is shown in Figure 1.

Evaluation of the pathogenicity of the variants

Nonsense, frameshift, canonical splice site variants and large insertions/deletions (affecting more than 5 amino acids) were classified as definitely pathogenic mutations. Missense variants and in-frame small insertions/deletions (accounting for less than 5 amino acids) were evaluated considering the evolutionary conservation of the amino-acid residue in orthologues [10], a number of *in silico* predictors (Sift, Polyphen, Mutation Taster), disease specific databases, and population data (ExAC database). Based on the score obtained in the sum of all these factors, missense variants were classified in highly likely pathogenic (score ≥11), likely pathogenic (score 5-10) , indeterminate (score 0-4), and highly likely neutral variants (score ≤-1) [11], [12]. All candidate pathogenic variants were validated by conventional PCR amplification and Sanger sequencing or by MLPA analysis. Segregation of these changes with the disease was assessed for all the available family members.

RESULTS

Sequencing statistics

Targeted sequencing of our kidney-disease gene panel demonstrated high depth of coverage and target coverage. A mean depth of coverage of 1393x was achieved for the 132 genes across all individuals. On average, 95.9% of the targeted region was covered to a minimum of 50x reads, 98.7% to a minimum of 20x and 99.7% to a minimum of 5x.

Pilot study

Mutations were identified in 115 of the 116 patients in the pilot study. A total of 134 of the 135 previously known mutations in their correct heterozygous/homozygous state were detected, for a sensitivity of 99%. The detected mutations included: 45 missense, 24 nonsense, 10 splicing, 25 small deletions, 9 small insertions, 4 deletion/insertion (indels), 14 large deletions and 3 large insertions. The only undetected mutation was a missense mutation in

PKD1 exon 1. No spurious pathogenic mutations were found in any of these samples. The results of the pilot study have been partially published for ADPKD and SRNS [8], [13].

Diagnostic yield in cystic IKD

Disease-causing mutations were identified in 52 of the 61 cystic patients including 27 of the 30 ADPKD patients (90%), 7 of the 8 ARPKD patients (88%), and 18 of the 23 TS patients (78%). Also, mutations in 6 of the 14 indeterminate cystic patients (43%) were detected (Figure 2). The mutations identified in cystic IKD patients are listed in Table 1.

Complex inheritance patterns were found in 3 ADPKD patients. Patient ADPKD-245 was diagnosed at 13 years of age with polycystic kidney disease, after multiple urinary tract infections. Renal ultrasound showed bilateral cortical renal cysts and he reached CKD stage 3 at 36 years old. His father had two large bilateral renal cysts (of 11 and 7 cm) removed by percutaneous puncture at 49 years of age. He also had a few bilateral simple renal cysts with normal renal function at 58 years old. Molecular analysis revealed two *PKD1* mutations: p.Q2641* and p.T1711I in the proband and only the p.T1711I in the father. These mutations were classified as definitely and likely pathogenic in the ADPKD mutation database, respectively. The T1711I mutation is a non-conservative change that alters an amino acid conserved throughout evolution to *Xenopus tropicalis* and is predicted to be highly likely pathogenic. Patient ADPKD-192 had a clinical diagnosis of ADPKD with CKD stage 5 at 44 years old. We detected two missense mutations in *PKD1* gene: the p.L727P, previously described as pathogenic mutation in the literature [11], and the p.R4007C, reported in the ADPKD mutation database as highly likely pathogenic. Both mutations are non-conservative changes that affect an evolutionarily conserved amino acid and are bioinformatically predicted to be highly likely pathogenic. Unfortunately, no more family members were available in this family to discern if both mutations were in *cis* or in *trans*. Patient ADPKD-216 had a clinical diagnosis of ADPKD and reached ESRD at 51 years of age. He carried the *PKD2* truncating mutation p.S74Pfs*43 together with the non-conservative *PKD1* missense change p.E1811K, considered of indeterminate clinical significance in the ADPKD mutation database. This E1811 amino acid is conserved in orthologues to *Danio rerio* except in *Canis lupus*, which has a conservative change at this position. The p.E1811K change is predicted to be damaging by SIFT and Polyphen but benign by Mutation taster, and it has been reported in ExAC database (MAF=0.0001).

Mosaicism was found in 3 TS patients. Patient TS-021 presented cortical tubers, facial angiofibromas, renal angiomyolipomas and renal cysts while her 38-year-old son had cortical tubers, mental retardation, epilepsy, facial angiofibromas, cardiac rhabdomyomas at infancy

and renal angiomyolipomas. The p.P1675L mutation was identified at mutant allele frequency of 14% (in 246 of the 5024 reads) in the mother and in heterozygous state in her son. This mutation has been previously published in the literature and is predicted to be highly likely pathogenic [14]. Patient TS-032 had cortical tubers, cardiac rhabdomyoma at infancy, facial angiofibromas and encroaching renal angiomyolipomas. Mutational analysis revealed the previously reported p.R1743W mutation in 15% (127 of the 722 reads) [15]. Patient TS-040 presented hypomelanotic macules, shagreen patch, facial angiofibromas and a small renal angiomyolipoma. The definitively pathogenic mutation p.T635Rfs*52 in the *TSC1* gene was found at mutant allele frequency of 9% (in 190 of the 2109 reads).

Mutations in genes included as differential diagnosis of cystic IKD were found in 6 patients that came to our laboratory with an indeterminate clinical diagnosis. *NPHP3* mutations were found in two fetuses. Patient NPHP3-001 was referred to our laboratory with a clinical suspicion of ARPKD because prenatal ultrasound revealed bilateral enlarged polycystic kidneys and anhydramnios. Patient NPHP3-002 resulted in a termination of pregnancy in the 21th week of gestation due to oligohydramnios. Renal histology showed renal cystic dysplasia and a pancreatic hamartoma.

Patient HNF1B-065 had renal cysts, bilateral hyperechogenic kidneys and poor cortico-medullary differentiation at 19 years of age suggestive of ADPKD with no family history of renal disease. Our approach identified a deletion of the whole *HNF1B* gene and the *PKD1* p.G1902R mutation. The *PKD1* mutation was predicted to be pathogenic by the *in silico* predictors, but was present in the ExAC database (MAF=0.00017). A putative contribution of this *PKD1* variant cannot be excluded. None of these variants were present in his mother and the father's sample was not available.

A definitively disease-causing mutation in the *OFD1* gene was found in patient ADPKD-138, a 35-year-old Japanese woman presenting with multiple renal cysts bilaterally and hyperechogenic kidneys on renal ultrasound.

Mutations in *PAX2* gene were found in 2 patients. In patient HNF1B-31 elevated serum creatinine was detected in the context of growth failure at 17 months. Renal ultrasound showed bilateral corticomedullary microcysts with increased renal echogenicity. She also presented proteinuria, microalbuminuria with CKD stage 2 at 10 years old. Mutation analysis revealed a missense mutation predicted to be highly likely pathogenic. Her mother had CKD stage 3 with proteinuria but she could not be analyzed. Patient HNF1B-030 presented with nephrotic-range proteinuria, hypercholesterolemia and CKD at 3 years old. No

hypoalbuminemia, edemas or hematuria were detected. Hypercalciuria was repeatedly found and glycosuria without hyperglycemia was occasionally detected. Renal ultrasound revealed slightly diminished kidneys, increased renal echogenicity and poor corticomedullary differentiation. Mutation analysis revealed a definitively pathogenic mutation in the *PAX2* gene. There was no family history of renal disease in this family.

Diagnostic yield in glomerular IKD

A genetic diagnosis was achieved in 20 of the 29 glomerular patients including 18 of the 22 AS patients (82%), 2 of the 5 SRNS patients (40%), and the 2 patients with HANAC (Figure 2). The mutations identified in glomerular IKD patients are listed in Table 2.

Structural variants in the AS genes were detected in 3 patients and consisted of a duplication of *COL4A5* exons 38-41 (patient AS-258), a duplication of *COL4A3* exons 30-35 (patient AS-187), and a whole *COL4A3* deletion (patient SRNS-280), all in heterozygous state.

Complex inheritance with mutations in 2 collagen type IV genes was found in 2 patients. Patient AS-115 was a 26-year-old woman with hipoacusia and nephrotic range proteinuria. Her mother and uncle also had microhematuria, proteinuria, and hipoacusia. Molecular analysis revealed that the three patients carried the *COL4A4* p.(G1451R) mutation together with the *COL4A3* p.(R1609Q) mutation. These novel non-conservative mutations affect an evolutionary conserved amino acid and are predicted to be highly likely pathogenic. Patient AS-252 presented with microhematuria and normal renal function at 47 years old. She carried the *COL4A3* truncating mutation p.T781_G783del and the *COL4A4* p.R1682Q previously described as pathogenic by Rana [16] and predicted to be highly likely pathogenic. Her brother had microhematuria at 48 years of age and progressed to CKD stage IV at 54, unfortunately, his DNA was not available.

Mutations in unexpected genes were identified in 2 SRNS patients. Patient SRNS-413 presented with SRNS at 3 years of age and was the only child of consanguineous parents. He partially responded to steroid treatment but moderate proteinuria persisted even with the immunosuppressive treatment. The renal biopsy showed minimal change disease and he had also celiac disease. We identified the homozygous missense mutation p. S1947Y in the *CUBN* gene. This mutation is a conservative change that alters an amino acid conserved throughout the evolution to *Caenorhabditis elegans,* it is predicted to be damaging by SIFT and polyphen but benign by Mutation taster and it has been reported in ExAC database (MAF=0.0003), resulting in a score consistent with a likely pathogenic variant. Patient SRNS-426 presented

with nephrotic range proteinuia and hypertension at 41 years old and his renal biopsy showed FSGS. Compound heterozygous missense mutations in the *NPHS1* gene, typical of congenital onset, were identified. Both mutations (p.V834F and p.R976S) had been previously reported [17], [18].

DISCUSSION

Annually, our laboratory receives around 100 unrelated patients for genetic diagnosis of cystic and glomerular IKD. Considering that most cystic and glomerular IKD are rare diseases, massive parallel sequencing of a disease-specific gene panel is not feasible to provide genetic diagnosis in a reasonable turn-over time. Thus, we aimed to develop a kidney-disease gene panel of 132 genes as a global tool for genetic diagnosis of cystic and glomerular IKD.

The high sensitivity and specificity obtained in our pilot study demonstrate the suitability of our kidney-disease gene panel for genetic diagnosis. All the mutations in the validation cohort were detected except a missense mutation in *PKD1* exon 1. Targeted sequencing of *PKD1* exon 1 with an adequate read depth is specially challenging due to its high GC content [13]. Thus, Sanger sequencing of this exon has to be performed in negative patients with no enough depth of coverage in this region.

Our results contribute to expand the phenotypic spectrum associated with low-frequently mutated IKD genes, some of them considered in the differential diagnosis of IKD. *NPHP3* mutations were identified as causative of adolescent onset NPHP-RC but childhood onset and even neonatal presentation have been described [19], [20]. This neonatal presentations can resemble ARPKD with enlarged kidneys and oligohydramnios [2], similar to the 2 fetuses presented here. The renal cystic disease found in oral-fascia-digital syndrome cannot always be distinguished from that of ADPKD by imaging techniques, such as in ADPKD-138 patient. Mutations in *PAX2* gene have been associated with CAKUT as part of a renal coloboma syndrome [21] but also with adult-onset FSGS [22]. Thus, the phenotype associated with *PAX2* mutations might have cystic and glomerular affectations, as patients HNF1B-31 and HNF1B-030. Similarly, CUBN mutations were initially described as causative of Imerslund-Gräsbeck syndrome, a form of congenital megaloblastic anemia due to vitamin B_{12} deficiency that in approximately half of patients present with proteinuria [23]. However, one family with nephrotic range proteinuria without the megaloblastic anemia, as the patient presented here, has also been reported [24].

Mosaic mutations in the TS have been reported in 6% and 58% of the TS patients with no mutations identified [25], [26], respectively. Based on these studies the estimated frequency of mosaic mutations in TS patients would be less than 1% [25] or 6-9% [26]. Our results support a high frequency of mosaic mutations in TS patients, that may be undetected if no high depth of coverage is achieved.

Structural variants in *COL4A3* were detected in 2 patients, suggesting that this type of genetic variants could account for a non-negligible proportion of ADAS cases. To the best of our knowledge large deletions or duplications in *COL4A3* and *COL4A4* genes had only been in 6 patients consisting of 3 deletions and 2 duplications in *COL4A3,* and 1 deletion in *COL4A4* [27]. Further studies including CNV analysis in large cohorts are needed to establish the proportion of patients carrying these types of mutations.

Complex inheritance patterns in ADPKD patients involve incompletely penetrant or hypomorphic *PKD1* alleles that can cause mild cystic disease when inherited alone or modulate the severity of the cystic disease in patients with another mutation [28], [29]. Consistently, our two patients with compound heterozygous *PKD1* mutations (ADPKD-245 and ADPKD-192) presented a more severe phenotype than that expected. Also, patient ADPKD-216 reached ESRD at 51 years old, which is extremely early for *PKD2* mutated patients. Thus, the missense *PKD1* mutation, although considered of indeterminate clinical significance, is likely to aggravate the phenotype in this patient.

Similarly, digenic inheritance in AS has recently been published. Patients with heterozygous mutations in two genes were reported to have more severe phenotypes than those with a single mutation. However, a high phenotypic variability among the probands of the different families was reported with ages at onset of hematuria ranging from childhood to adulthood [30]. Here, two patients carrying combined heterozygous *COL4A3* and *COL4A4* disease-causing mutations were identified. Patient AS-115 and her family members presented a more severe phenotype than that expected for the AD form with a childhood onset of the disease and hipoacusia. In contrast, the phenotype of patient AS-252 is in accordance with ADAS or collagen type IV (α3α4) nephropathy. It is tempting to speculate that her brother might carry the same genotype as her, likely explaining his rapid progression although it could not be confirmed. Therefore, digenic inheritance may explain the intrafamiliar variability but much more genetic and environmental factors seem to play a role in the interfamiliar variability, especially in patients with adult onset disease.

The high throughput of massive parallel sequencing makes the number of genes and their length no longer a barrier for genetic analysis. In terms of costs, our kidney panel approach is estimated to save 40% of costs per sample compared with sequential Sanger sequencing and MLPA analysis of candidate genes. However, interpretation of the amount of data generated is challenging. Large-scale sequencing projects have revealed the existence of many dozens of genetic variants predicted to severely disrupt protein-coding genes in the genomes of apparently healthy individuals, even in homozygous state [31]. Thus, the assumption that variants that severely disrupt gene function are likely to be disease-causing must be cautiously taken. Even more complex are patients with complex inheritance patterns or digenic inheritance in whom the form of genetic transmission will depend on whether the mutant alleles of the two genes are on the same parental allele (*cis* configuration) or on different ones (*trans* configuration). In this context, genetic analysis of the parents as well as other affected and unaffected relatives, detailed clinical information, and experienced geneticists specialized in the diseases analyzed are essential to provide an accurate genetic diagnosis and genetic counseling.

In conclusion, massive parallel sequencing of our kidney-disease gene panel is a comprehensive, efficient and cost-effective tool for genetic diagnosis of cystic and glomerular IKD. Our approach improves the genetic diagnosis by detecting all types of genetic variation, including structural variants and mosaic mutations, as well as complex inheritance patterns.

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Figure 1: Genetic testing strategy. Schematic visualization of the workflow used for genetic diagnosis of cystic and glomerular IKD.

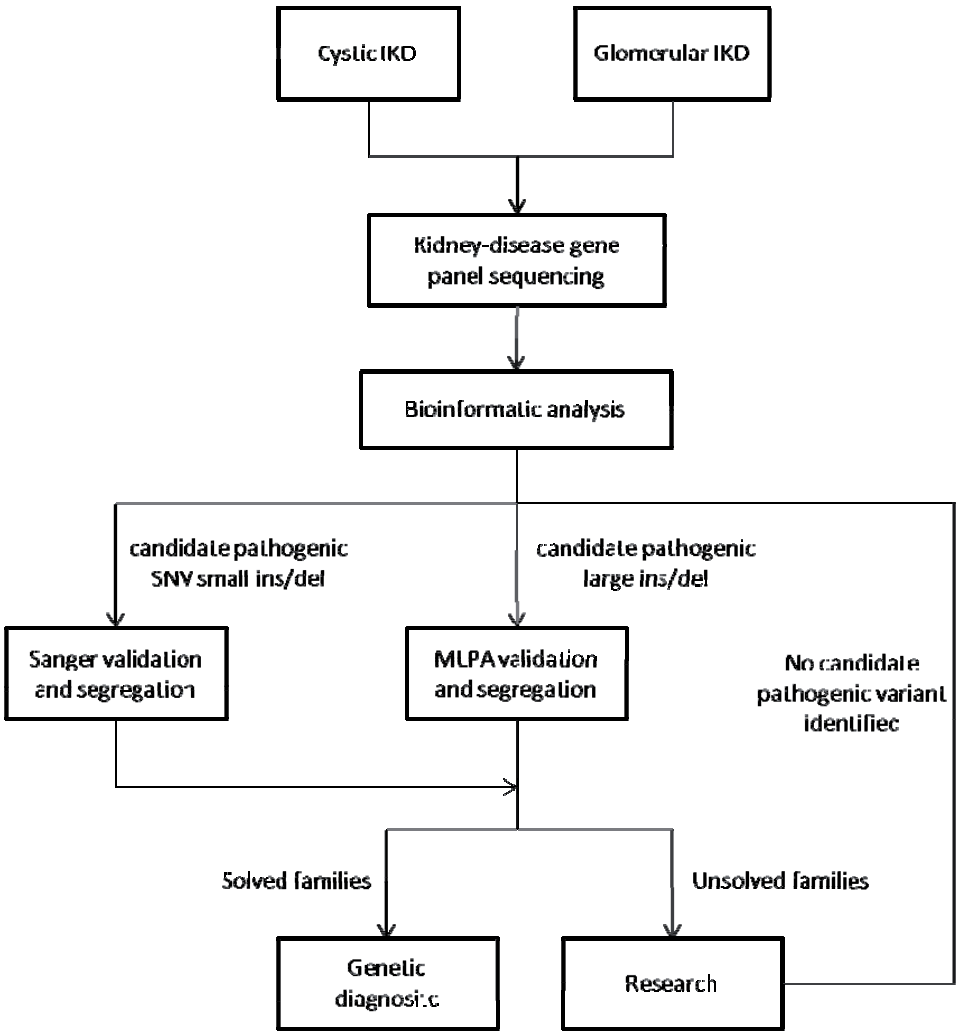

Figure 2: Percentage of diagnostic patients with a molecular diagnosis for each group of cystic and glomerular IKD. ADPKD, autosomal dominant polycystic kidney disease; ARPKD, autosomal recessive polycystic kidney disease; AS, alport syndrome; HANAC, Hereditary angiopathy with nephropathy, aneurysms, and muscle cramps; IKD, inherited kidney diseases; SRNS, steroid-resistant nephrotic syndrome; TS, tuberous sclerosis.

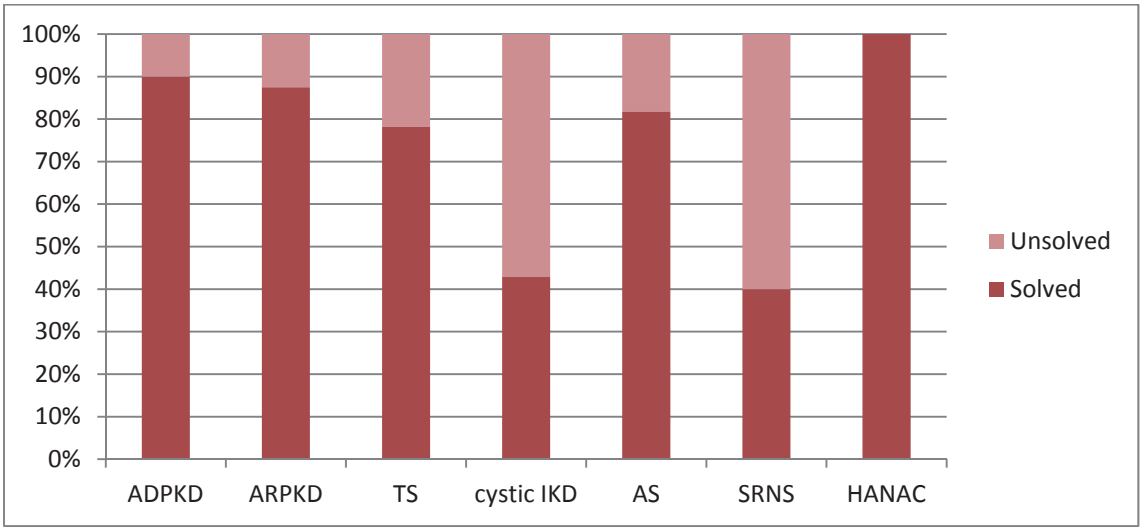

Table 1. Mutations identified in diagnostic patients with cystic IKD.

Patient	Gene	Mutation	Exon (intron)	Predicted pathogenicity	Inheritance pattern
ADPKD					
ADPKD-215	*PKD1*	c.323A>G p.(Glu108Gly)	3	Highly likely pathogenic	AD
ADPKD-223	*PKD1*	c.380T>G p.(Phe127Cys)	4	Likely pathogenic	AD
ADPKD-228	*PKD1*	c.566C>G p.(Ser189*)	5	Definitely Pathogenic	AD
ADPKD-232	*PKD1*	c.1202-A>T p.(?)	(5)	Definitely Pathogenic	AD
ADPKD-212	*PKD1*	c.1350delC p.(Ala451Profs*14)	6	Definitely Pathogenic	AD
ADPKD-230	*PKD1*	c.1522T>C (p.Cys508Arg)	7	Highly likely pathogenic	AD
ADPKD-246	*PKD1*	c.2534T>C p.(Leu845Ser)	11	Highly likely pathogenic	AD
ADPKD-235	*PKD1*	PKD1: c.3296-1G>T (IVS14-1G>T)	(14)	Definitely Pathogenic	AD
ADPKD-243	*PKD1*	c.3983G>C p.(Trp1328Ser)	15	Highly likely pathogenic	AD
ADPKD-231	*PKD1*	c.6493C>T p.(Gln2165*)	15	Definitely Pathogenic	AD
ADPKD-244	*PKD1*	c.7108T>C p.(C2370R)	17	Highly likely pathogenic	AD
ADPKD-189	*PKD1*	c.7622C>T p.(Pro2541Leu)	19	Highly likely pathogenic	AD
ADPKD-220	*PKD1*	c.8895_8896delAG p.(Glu2966Valfs*6)	24	Definitely Pathogenic	AD
ADPKD-251	*PKD1*	c.9965_9966del p.(Thr3322Serfs*67)	30	Definitely pathogenic	AD
ADPKD-247	*PKD1*	c.11249_11263del p.(Arg3750_Leu3754del5)	39	Definitely pathogenic	AD
ADPKD-001	*PKD1*	c.11524T>C p.(Trp3842Arg)	41	Highly likely pathogenic	AD
ADPKD-201	*PKD1*	c.11629_11639delins11; p.(G3877_L3880delinsRARP)	42	Definitely Pathogenic	AD
ADPKD-226	*PKD1*	c.11732_11734delCCG p.(Ala3911del)	43	Definitely Pathogenic	AD
ADPKD-237	*PKD1*	c.12076C>T p.(Leu4026Phe)	44	Highly likely pathogenic	AD
ADPKD-242	*PKD1*	c.12167G>A p.(Trp4056*)	45	Definitely Pathogenic	AD
ADPKD-249	*PKD2*	c.717C>A:p.Tyr239X	3	Definitely pathogenic	AD
ADPKD-224	*PKD2*	c.1249C>T p.(Arg417*)	5	Definitely Pathogenic	AD
ADPKD-250	*PKD2*	c.2161dupA p.(Thr721Asnfs*4)	11	Definitely pathogenic	AD
ADPKD-236	*PKD2*	PKD2: c.2533C>T (p.Arg845*)	14	Definitely Pathogenic	AD
ADPKD-245	*PKD1*	c.7921C>T p.(Gln2641*)	21	Definitely Pathogenic	Complex
	PKD1	c.5132C>T p.(Thr1711Ile)	15	Highly likely pathogenic	
ADPKD-192	*PKD1*	c.2180T>A p.(Leu727Gln)	11	Highly likely pathogenic	Complex
	PKD1	c. 12019C>T p.(Arg4007Cys)	44	Likely pathogenic	
ADPKD-216	*PKD2*	c.219delC p.(Ser74Profs*43)	1	Definitely Pathogenic	Complex
	PKD1	c.5431G>A p.(Glu1811Lys)	15	Indeterminate	
ARPKD					
ARPKD-099	*PKHD1*	c.1305delG p.(Thr436Profs*26)	16	Definitely Pathogenic	AR
	PKHD1	c.1529delG p.(Gly510Alafs*25)	17	Definitely Pathogenic	
ARPKD-100	*PKHD1*	c.5903T>G p.(Ile1968Ser)	36	Highly likely pathogenic	AR
	PKHD1	c.9689del p.(Asp3230Valfs*34)	58	Definitely pathogenic	
ARPKD-101	*PKHD1*	c.8893T>C p.(Cys2965Arg)	57	Highly likely pathogenic	AR
	PKHD1	c.8893T>C p.(Cys2965Arg)	57	Highly likely pathogenic	
ARPKD-103	*PKHD1*	c.842G>A p.(Gly281Glu)	12	Highly likely pathogenic	AR
	PKHD1	c.1529delG p.(Gly510Alafs*25)	17	Definitely pathogenic	
ARPKD-04*	*PKHD1*	c.10891delT p.(Tyr3631Ilefs*11)	32	Definitely pathogenic	AR
ARPKD-35	*PKHD1*	c.107C>T p.(Thr36Met)	3	Likely pathogenic	AR
	PKHD1	c.8114delG p.(Gly2705Valfs*11)	51	Definitely Pathogenic	

Patient	Gene	Mutation	Exon (intron)	Predicted pathogenicity	Inheritance pattern
ARPKD					
ARPKD-085	*PKHD1*	c.2810G>A p.(Trp937*)	26	Definitely pathogenic	AR
	PKHD1	c.5125C>T p.(Leu1709Phe)	32	Indeterminate	
Tuberous sclerosis					
TS-038	*TSC2*	c.275A>T p.(Glu92Val)	4	Likely pathogenic	AD
TS-007	*TSC2*	c.768C>G p.(Cys256Trp)	8	Highly likely pathogenic	AD
TS-018	*TSC2*	c.1099C>T p.(Arg367Trp)	11	Highly likely pathogenic	AD
TS-023	*TSC2*	c.1916G>A p.(Arg639Gln)	18	Likely pathogenic	AD
TS-020	*TSC2*	c.2713C>T p.(Arg905Trp)	24	Highly likely pathogenic	AD
TS-004	*TSC2*	c.2824G>T p.(Glu942*)	25	Definitely Pathogenic	AD
TS-035	*TSC2*	c.3451_6455delinsCCCC p.(Gly1151Profs*40)	28	Definitely Pathogenic	AD
TS-033	*TSC2*	c.4515C>G p.(Tyr1505*)	35	Definitely Pathogenic	AD
TS-021	*TSC2*	c.5024C>T p.(Pro1675Leu)	39	Highly likely pathogenic	Mosaic
TS-037	*TSC2*	c.5161-1G>C p.(?)	41	Definitely Pathogenic	AD
TS-032	*TSC2*	c.5227C>T p.(Arg1743Trp)	41	Highly likely pathogenic	Mosaic
TS-005	*TSC2*	c.5228G>A p.(Arg1743Gln)	41	Highly likely pathogenic	AD
TS-006	*TSC2*	c.5228G>A p.(Arg1743Gln)	41	Highly likely pathogenic	AD
TS-013	*TSC1*	c.278T>A p.(Leu93Gln)	5	Highly likely pathogenic	AD
TS-030	*TSC1*	c.338delT p.(Leu113Cysfs*5)	5	Definitely Pathogenic	AD
TS-017	*TSC1*	c.508+1G>A p.(?)	(6)	Definitely Pathogenic	AD
TS-040	*TSC1*	c.1904_1905del p.(Thr635Argfs*52)	15	Definitely Pathogenic	Mosaic
TS-015	*TSC1*	c.2268_2271delAGAA p.(Lys756Asnfs*16)	18	Definitely Pathogenic	AD
Indeterminate cystic IKD					
NPHP3-001	*NPHP3*	c.1084_1087delTTAG p.(Val363Phefs*6)	6	Definitely Pathogenic	AR
	NPHP3	c.2694-2_2694-1del p.(?)	(19)	Definitely Pathogenic	
NPHP3-002	*NPHP3*	c.434_437delAAAG p.(Glu145Valfs*3)	2	Definitely Pathogenic	AR
	NPHP3	c.434_437delAAAG p.(Glu145Valfs*3)	2	Definitely Pathogenic	
HNF1B-065	*HNF1B*	whole gene deletion	1-8	Definitely Pathogenic	AD
PAX2-003	*PAX2*	c.70G>T p.(Gly24Trp)	2	Highly likely pathogenic	AD
PAX2-004	*PAX2*	c.495_496insCCCTGA p.(Val166_Val431delinsPro)	4	Definitely Pathogenic	AD
OFD1-001	*OFD1*	c.840_844del p.(Lys280Asnfs*27)	9	Definitely Pathogenic	AD

*Mother of an affected child, only one mutation expected

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; IKD, inherited kidney diseases.

Mutations on these genes were classified according to Genebank Accession numbers: NG_008617.1, NM_001009944.2 and NP_001009944.2 (PKD1); NG_008604.1, NM_000297.3 and NP_000288.1 (PKD2); NG_008753.1, NM_138694.3 and NP_619639.3 (PKHD1); NG_005895.1, NM_000548.4 and NP_000539.2 (TSC2); NG_012386.1, NM_000368.4 and NP_000359.1 (TSC1); NG_008130.1 , NM_153240.4 and NP_694972.3 (NPHP3); NG_013019.2, NM_000458.3 and NP_000449.1 (HNF1B); NG_008680.2, NM_000278.4 and NP_000269.3 (PAX2); NG_008872.1, NM_003611.2 and NP_003602.1 (OFD1).

Nomenclature of sequence variants in DNA and protein is in accordance with the Human Genome Variation Society guidelines (www.hgvs.org).

Table 2. Mutations identified in diagnostic patients with glomerular IKD.

Patient	Gene	Mutation	Exon (intron)	Predicted pathogenicity	Inheritance pattern
AS/ collagen type IV (α3α4) nephropathy					
AS-245	*COL4A5*	c.412delG:p.G138fs	7	Definitively pathogenic	XL
AS-224	*COL4A5*	c.C796T:p.R266X	14	Definitively pathogenic	XL
AS-257	*COL4A5*	c.2216C>A p.(Pro739His)	28	Likely pathogenic	XL
AS-230	*COL4A5*	c.G3319A:p.G1107R	37	Highly likely pathogenic	XL
AS-236	*COL4A5*	c.G3373A:p.G1125R	37	Highly likely pathogenic	XL
AS-258	*COL4A5*	EX 38-41 DUPLICATION	38-41	Definitely Pathogenic	XL
AS-248	*COL4A5*	c.3659G>A p.(Gly1220Asp)	41	Highly likely pathogenic	XL
AS-247	*COL4A5*	c.3884delT p.(Leu1295Cysfs*4)	42	Definitely Pathogenic	XL
AS-261	*COL4A5*	c.4887_4948del p.Ile1630Asnfs*10	50	Definitely Pathogenic	XL
AS-147	*COL4A3*	c.2275G>A p.(Gly759Arg)	30	Highly likely pathogenic	AR
		c.4981C>T p.(Arg1661Cys)	52	Highly likely pathogenic	
AS-189	*COL4A4*	c.2320G>C p.(Gly774Arg)	28	Likely pathogenic	AR
		c.4508delA p.(His1503Profs*49)	46	Definitely Pathogenic	
AS-256	*COL4A3*	c.1391G>T p.(Gly464Val)	22	Highly likely pathogenic	AD
AS-255	*COL4A3*	c.2384G>T p.(Gly795Val)	31	Highly likely pathogenic	AD
AS-187	*COL4A3*	exons 30-35 duplication	30-35	Definitely Pathogenic	AD
AS-277	*COL4A3*	whole gene deletion	1-52	Definitely Pathogenic	AD
AS-249	*COL4A4*	c.2733_2741del p.(Phe916_Gly918del	31	Definitely Pathogenic	AD
AS-252	*COL4A3*	c.2342_2350del p.(Thr781_Gly783del)	30	Definitely Pathogenic	Digenic
	COL4A4	c.5045G>A p.(Arg1682Gln)	48	Highly likely pathogenic	
AS-115	*COL4A4*	c.4351G>A p.(Gly1451Arg)	46	Highly likely pathogenic	Digenic
	COL4A3	c.4826G>A p.(Arg1609Gln)	51	Highly likely pathogenic	
SRNS/FSGS					
SRNS-413	*CUBN*	c.5840C>A p.(Ser1947Tyr)	39	Likely pathogenic	AR
	CUBN	c.5840C>A p.(Ser1947Tyr)	39	Likely pathogenic	
SRNS-426	*NPHS1*	c.2500G>T p.(Val834Phe)	18	Highly likely pathogenic	AR
	NPHS1	c.2928G>T p.(Arg976Ser)	22	Highly likely pathogenic	
HANAC					
HANAC-001	*COL4A1*	c.1181G>A p.(Gly394Asp)	21	Highly likely pathogenic	AD
HANAC-002	*COL4A1*	c.1493G>A p.(Gly498Asp)	24	Highly likely pathogenic	AD

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; AS, Alport syndrome; FSGS, focal segmental glomerulosclerosis; HANAC, Hereditary angiopathy with nephropathy, aneurysms, and muscle cramps; IKD, inherited kidney diseases; SRNS, steroid-resistant nephrotic syndrome.

Mutations on these genes were classified according to Genebank Accession numbers: NG_011977.1, NM_000495.4 and NP_000486.1 (COL4A5); NG_011591.1, NM_000091.4 and NP_000082.2 COL4A3, NG_011592.1, NM_000092.4 and NP_000083.3 (COL4A4); NG_008967.1, NM_001081.3 and NP_001072.2 (CUBN), NG_013356.2, NM_004646.3 and NP_004637.1 (NPHS1); NG_011544.2, NM_001845.5 and NP_001836.3 COL4A1.

Nomenclature of sequence variants in DNA and protein is in accordance with the Human Genome Variation Society guidelines (www.hgvs.org).

Supplementary Table 1. The 132 genes included in the kidney-disease gene panel

Gene	Disease association	Inheritance pattern	Accession nº
Cystic diseases			
PKD1	Autosomal dominant polycystic kidney disease	AD	NM_000296
PKD2	Autosomal dominant polycystic kidney disease	AD	NM_000297
PKHD1	Autosomal recessive polycystic kidney disease	AR	NM_138694
PRKCSH	Polycystic liver disease	AD	NM_001001329
SEC63	Polycystic liver disease	AD	NM_007214
NPHP1	Nephronophthisis-related ciliopathies	AR	NM_000272
INVS	Nephronophthisis-related ciliopathies	AR	NM_014425
NPHP3	Nephronophthisis-related ciliopathies	AR	NM_153240
NPHP4	Nephronophthisis-related ciliopathies	AR	NM_015102
IQCB1	Nephronophthisis-related ciliopathies	AR	NM_001023570
CEP290	Nephronophthisis-related ciliopathies	AR	NM_025114
GLIS2	Nephronophthisis-related ciliopathies	AR	NM_032575
RPGRIP1L	Nephronophthisis-related ciliopathies	AR	NM_001127897
NEK8	Nephronophthisis-related ciliopathies	AR	NM_178170
SDCCAG8	Nephronophthisis-related ciliopathies	AR	NM_006642
TMEM67	Nephronophthisis-related ciliopathies	AR	NR_024522
TTC21B	Nephronophthisis-related ciliopathies and FSGS	AR	NM_024753
WDR19	Nephronophthisis-related ciliopathies	AR	NM_025132
ZNF423	Nephronophthisis-related ciliopathies	AD/AR	NM_015069
CEP164	Nephronophthisis-related ciliopathies	AR	NM_014956
ANKS6	Nephronophthisis-related ciliopathies	AR	NM_173551
CC2D2A	Nephronophthisis-related ciliopathies	AR	NM_001080522
IFT172	Nephronophthisis-related ciliopathies	AR	NM_015662
CEP83	Nephronophthisis-related ciliopathies	AR	NM_016122
BBS1	Nephronophthisis-related ciliopathies	AR	NM_024649
BBS10	Nephronophthisis-related ciliopathies	AR	NM_024685
BBS2	Nephronophthisis-related ciliopathies	AR	NM_031885
BBS4	Nephronophthisis-related ciliopathies	AR	NM_001252678
BBS5	Nephronophthisis-related ciliopathies	AR	NM_152384
BBS7	Nephronophthisis-related ciliopathies	AR	NM_176824
BBS9	Nephronophthisis-related ciliopathies	AR	NM_001033604
BBS12	Nephronophthisis-related ciliopathies	AR	NM_001178007
ARL6	Nephronophthisis-related ciliopathies	AR	NM_032146
MKKS	Nephronophthisis-related ciliopathies	AR	NM_170784
TRIM32	Nephronophthisis-related ciliopathies	AR	NM_001099679
TTC8	Nephronophthisis-related ciliopathies	AR	NM_144596
WDPCP	Nephronophthisis-related ciliopathies	AR	NM_015910
MKS1	Nephronophthisis-related ciliopathies	AR	NM_001165927
TMEM216	Nephronophthisis-related ciliopathies	AR	NM_001173990
TCTN2	Nephronophthisis-related ciliopathies	AR	NM_001143850
UMOD	Autosomal dominant tubulointerstitial kidney disease	AD	NM_001008389
REN	Autosomal dominant tubulointerstitial kidney disease		NM_000537

Gene	Disease association	Inheritance pattern	Accession nº
Cystic diseases			
TSC1	Tuberous sclerosis	AD	NM_000368
TSC2	Tuberous sclerosis	AD	NM_000548
OFD1	Oral-fascia-digital syndrome I	AD	NM_003611
NOTCH2	Renal Cysts and Papillary Microadenomas	AD	NM_024408
VHL	Von Hippel-Lindau syndrome	AD	NM_000551
MET	Renal cell carcinoma, papillary, 1	unknown	NM_001127500
FLCN	Birt-Hogg-Dube syndrome	AD	NM_144997
SDHD	Pheochromocytoma	AD	NM_003002
SDHB	Pheochromocytoma	AD	NM_003000
CAKUT			
HNF1B	Renal cysts and diabetes syndrome	AD	NM_000458
PAX2	CAKUT	AD/AR	NM_003990
SALL1	CAKUT	AD	NM_002968
CHD1L	CAKUT	AD	NM_004284
ROBO2	CAKUT	AD	NM_001290040
EYA1	CAKUT	AD	NM_000503
RET	CAKUT	AD	NM_020975
GATA3	CAKUT	AD	NM_001002295
SIX1	CAKUT	AD	NM_005982
BMP7	CAKUT	AD	NM_001719
CDC5L	CAKUT	AD	NM_001253
SIX2	CAKUT	AD	NM_016932
SIX5	CAKUT	AD	NM_175875
BMP4	CAKUT	AD	NM_001202
SOX17	CAKUT	AD	NM_022454
UPK3A	CAKUT	AD	NM_006953
FGF20	CAKUT	AR	NM_019851
TNXB	CAKUT	AD	NM_019105
WNT4	CAKUT	AD	NM_030761
DSTYK	CAKUT	AD	NM_015375
TRAP1	CAKUT	AR	NM_016292
FREM2	CAKUT	AD	NM_207361
ITGA8	CAKUT	AR	NM_003638
SEMA3A	CAKUT	AD	NM_006080
ACE	CAKUT	AR	NM_000789
GRIP1	CAKUT	AR	NM_021150
FRAS1	CAKUT	AR	NM_025074
DCHS2	CAKUT candidate	unknown	NM_017639
FAT1	CAKUT candidate	unknown	NM_005245
FAT4	CAKUT candidate	unknown	NM_001291303
LGR4	CAKUT candidate	unknown	NM_018490
GDF11	CAKUT candidate	unknown	NM_005811
ROBO1	CAKUT candidate	unknown	NM_002941
HGF	CAKUT candidate	unknown	NM_000601

Gene	Disease association	Inheritance pattern	Accession nº
CAKUT			
TRPS1	CAKUT candidate	unknown	NM_014112
FMN1	CAKUT candidate	unknown	NM_001277313
FAT3	CAKUT candidate	unknown	NM_001008781
DAAM1	CAKUT candidate	unknown	NM_014992
Glomerular diseases			
COL4A5	Collagen type IV nephropathy	XL	NM_000495.4
COL4A3	Collagen type IV nephropathy	AD/AR	NM_000091.4
COL4A4	Collagen type IV nephropathy	AD/AR	NM_000092.4
COL4A6	Collagen type IV nephropathy with leiomyomatosis	XL	NM_001287758
COL4A1	HANAC	AD	NM_001845
NPHS1	SRNS	AR	NM_004646.2
NPHS2	SRNS	AR	NM_014625.2
PLCE1	SRNS	AR	NM_016341.3
CD2AP	FSGS	AD/AR	NM_012120.2
PTPRO	SRNS	AR	NM_030667.2
MYO1E	SRNS	AR	NM_004998.3
ARHGDIA	SRNS	AR	NM_001185077
ADCK4	SRNS	AR	NM_024876
CRB2	SRNS	AR	NM_173689
WT1	SRNS, Denys-Drash syndrome	AD	NM_000378.4
ACTN4	SRNS, FSGS	AD	NM_004924.4
TRPC6	SRNS, FSGS	AD	NM_004621.5
INF2	SRNS, FSGS	AD	NM_001031714.3
LMX1B	SRNS	AD	NM_001174146.1
ARHGAP24	SRNS, FSGS	AD	NM_001025616.2
ANLN	SRNS	AD	NM_018685
LAMB2	SRNS	AR	NM_002292.3
SMARCAL1	SRNS	AR	NM_001127207.1
MT-TL1	SRNS	mitochondrial	ENSG00000209082
COQ2	SRNS	AR	NM_015697.7
COQ6	SRNS	AR	NM_182476.2
PDSS2	SRNS	AR	NM_020381.3
SCARB2	SRNS	AR	NM_005506.3
ITGA3	SRNS	AR	NM_002204.2
ITGB4	SRNS	AR	NM_000213
CUBN	SRNS	AR	NM_001081.3
CFH	SRNS	AR	NM_000186.3
NEIL1	SRNS	AR	NM_001256552.1
EMP2	SRNS	AR	NM_001424
DGKE	membranoproliferative glomerulonephritis	AR	NM_003647
NXF5	FSGS and heart block disorder	XL	NM_032946
XPO5	SRNS	AR	NM_020750
NUP107	SRNS	AR	NM_020401
PODXL	FSGS candidate	AD	NM_001018111

Gene	Disease association	Inheritance pattern	Accession nº
Glomerular diseases			
LAMA5	FSGS candidate	unknown	NM_005560
APOL1	Risk allele FSGS	unknown	NM_145343
MYH9	Risk allele FSGS	unknown	NM_002473
GLA	Fabry disease	XL	NM_000169.2

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; CAKUT, congenital anomalies of the kidney and urinary tract; FSGS, focal segmental glomerulosclerosis; HANAC, Hereditary angiopathy with nephropathy, aneurysms, and muscle cramps; SRNS, steroid-resitant nephrotic syndrome.

DISCUSSION

IDIOPATHIC NEPHROTIC SYNDROME

Diseases of the GFB are characterized by barrier disruption with consequent proteinuria and/or microhematuria in urine. Proteinuria at presentation is indicative of podocyte damage whereas microhematuria points to a GBM defect. As disease progresses, proteinuria is present in most of cases. Persistent proteinuria leads to chronic renal failure and, ultimately, ESRD. Causes of barrier disruption range from congenital disorders associated with genetic mutations to acquired diseases linked to several inflammatory or metabolic disturbances, which may specifically target the glomerulus or be part of a wider systemic illness.

This broad range of causes is encompassed in the different forms of INS. CNF is a pure genetic disease. SRNS is considered to have a genetic cause whereas SSNS/SDNS or SRNS with immunosuppressive response or relapse after kidney transplantations are considered to have an underlying immune defect. Finally, IMN is an organ-specific autoimmune disease. In this thesis, the implication of genetics in the pathogenesis and clinical outcome of the different forms of INS was studied.

IDIOPATHIC MEMBRANOUS NEPHROPATHY: THE IMPORTANCE OF GENETICS

The discovery of PLA2R1 as a major antigen in IMN was a breakthrough in our understanding of the pathogenesis of the disease and established IMN as an autoimmune disease (Beck *et al.,* 2009). However, genetic susceptibility factors also play a role in the development of the disease. A genome-wide association study involving three independent cohorts (British, Dutch and French cohorts) identified noncoding SNPs within *HLA-DQA1* and *PLA2R1* genes as strong risk factors for IMN development. Interestingly, carrying the combination of the risk alleles for both SNPs had an additive effect. Patients carrying both risk alleles had an odds ratio close to 80 compared to individuals who had only protective alleles (Stanescu *et al.,* 2011). In this thesis, the association of *HLA-DQA1* (rs2187668) and *PLA2R1* (rs4664308) with IMN susceptibility was confirmed in a Spanish cohort. The combination of high-risk genotypes for both SNPs was also associated with a higher risk of IMN (Bullich *et al.,* 2014). Whether these genetic susceptibility factors also apply to patients with THSD7A-associated IMN remains to be determined (Tomas *et al.,* 2014).

The significant association found not only at the SNP level but also at the haplotype level raised the hypothesis that rare genetic variants within the coding region of this *PLA2R1* gene may contribute to antibody formation (Stanescu *et al.,* 2011; Coenen *et al.,* 2013). This possibility was also supported by the fact that autoantibody response in IMN is restricted to a conformation-dependent epitope of PLA2R1 (Beck *et al.,* 2009; Llorca 2008). Thus, causal or predisposing variants of the *PLA2R1* sequence would modify the structure of the protein to give rise to an epitope that would fit the antigen-presenting groove of a complementary, also causal or predisposing variant to HLA genes. Coenen *et al.* found no evidence to support this hypothesis, since in a cohort of 95 IMN patients, only 9 patients carried rare sequence variants in the *PLA2R1* gene, and only 4 of the 9 patients were among the 60 patients who presented autoantibodies against PLA2R1 (Coenen *et al.,* 2013).

Curiously, the genetic variants found to be associated with IMN are relatively common variants even though IMN is a rare disease. To explain this inconsistency, it has been speculated that the combination of relatively common variants may result in rare haploblocks that confers susceptibility to IMN. The rare confluence of relatively common events (genetic variants or environmental factors) might constitute the pathogenic trigger in IMN (Coenen *et al.,* 2013). More studies are needed to explain how alleles in the *HLA-DQA1* and *PLA2R1* loci interact with each other to increase susceptibility to IMN.

CNVs in the *FCGR* locus have been associated with susceptibility to several autoimmune diseases, such as antiglomerular basement membrane disease (Zhou *et al.,* 2010), glomerulonephritis in systemic lupus erythematosus, and primary Sjögren's syndrome (Aitman *et al.,* 2006; Fanciulli *et al.,* 2007). The genes included in this locus encode Fc receptors for IgG that have a crucial role in the generation of a well-balanced immune response. *FCGR3A* is mainly expressed by natural killer cells and participates in antibody-dependent cell-mediated cytotoxicity, whereas *FCGR3B* is predominantly expressed by neutrophils and is involved in immune complex clearance (Nimmerjahn & Ravetch 2008). In this thesis, the putative association of *FCGR3A* and *FCGR3B* CNVs with IMN was analyzed. We hypothesized that low *FCGR3A* and *FCGR3B* CN could decrease antibody-dependent cell-mediated cytotoxicity and immune complex clearance, respectively. No statistically significant differences in the CN profile of *FCGR3A* and *FCGR3B* between IMN patients and controls were found, indicating no contribution of *FCGR3B* CNVs to IMN susceptibility. However, control patients showed a trend to low *FCGR3A* whereas none of the IMN patients had low *FCGR3A* CN. Additional studies with larger cohorts may help to clarify this putative relationship (Bullich *et al.,* 2014).

The extremely variable clinical course of IMN and the controversial immunosuppressive therapy make treatment decisions challenging. The typical dilemma for nephrologists after establishing a diagnosis of IMN is whether to maintain a conservative treatment (a low-salt diet, RAAS blockers, and diuretics), waiting for a spontaneous remission, or to start with immunosuppressive therapy (Praga & Rojas-Rivera 2012). Reliable predictors for the spontaneous remission and for the potential need or the intensity of immunosuppressive therapy would be a very helpful tool for treatment decisions (Hoxha *et al.,* 2011). In this thesis, the putative role of SNPs in *HLA-DQA1* (rs2187668) and *PLA2R1* (rs4664308) and CNVs in *FCGR3B* gene as predictors for spontaneous remission, immunosuppressive therapy response, and renal function decline was studied.

Spontaneous remission was not significantly associated with any of the genetic variants analyzed in this study. However, either high or low *FCGR3B* CN was found in 18% of non-spontaneous remission patients but only in 4% of spontaneous remission patients, suggesting that alterations in *FCGR3B* CN could hinder spontaneous remission. *FCGR3B* CN could alter the balance between Fc receptors, disrupting the tightly regulated immune system (McKinney & Merriman 2012) and impeding achievement of spontaneous remission (Bullich *et al.,* 2014).

Regarding response to immunosuppressive therapy and renal function decline, our results showed that the risk genotypes for IMN development (A/A or A/G for *HLA-DQA1* and A/A for *PLA2R1*) predicted a positive response to immunosuppressive therapy and protection to renal function decline. These predictive values increased when adjusting for baseline proteinuria. To the best of our knowledge, this association is the first found between genetic variants and clinical outcome in IMN (Bullich *et al.,* 2014). In addition, among individuals carrying risk alleles, 73% had anti-PLA2R antibodies and 75% expressed PLA2R in glomeruli. In contrast, none of the individuals carrying the protective genotypes of both genes had anti-PLA2R antibodies and glomerular expression of PLA2R was weak or absent. Therefore, individuals carrying risk alleles have a predisposition to anti-PLA2R autoantibody generation and PLA2R expression (Lv *et al.,* 2013). Based on all these results, carrying risk alleles in *HLA-DQA1* and *PLA2R1* genes is a susceptibility factor for anti-PLA2R autoantibody generation and, in consequence, IMN development, but also a predictor of response to immunosuppressive therapy. In patients carrying the protective genotypes other genetic and environmental factors could contribute to IMN development, likely explaining their low response to immunosuppressive therapy.

Our results indicate that the *HLA-DQA1* (rs2187668) and *PLA2R1* (rs4664308) genotypes could add some predictive value to the currently used clinical and histologic markers. Recently the potential role of measuring PLA2R1 antibodies for clinical practice was suggested by studies showing that the presence of PLA2R antibodies supported a diagnosis of IMN (Beck *et al.,* 2009; Hoxha *et al.,* 2011; Hofstra *et al.,* 2012;). Furthermore, a correlation between the serum anti PLA2R levels and clinical disease activity was reported (Beck *et al.,* 2009; Hofstra *et al.,* 2011). Anti-PLA2R levels were high when patients had nephrotic range proteinuria, were substantially decreased or absent in the setting of spontaneous or treatment-induced remission, and were again increased in association with relapse (Hofstra *et al.,* 2011). In fact, the disappearance of antibodies preceded and predicted subsequent decrease of proteinuria, (Beck *et al.,* 2011) and high titers of antibodies were associated with a low likelihood of spontaneous remission (Hofstra *et al.,* 2012). Based on these findings, detection and measuring the levels of anti-PLA2R1 has been proposed to monitor disease activity, treatment efficacy and assist therapeutic decisions (Hofstra *et al.,* 2011; Bech *et al.,* 2014). Moreover, several commercially tests have been developed using indirect fluorescent-antibody technique (IFA) or ELISA and are currently commercially available (Debiec & Ronco 2011a; Hoxha *et al.,* 2011; Kanigicherla *et al.,* 2013; Dahnrich *et al.,* 2013).

THE KIDNEY-DISEASE GENE PANEL: THE INTEGRATIVE APPROACH

The high genetic heterogeneity and phenotypic variability of cystic and glomerular IKD makes genetic testing by traditional Sanger sequencing expensive and time-consuming. Genetic testing by Sanger sequencing consists of the sequential analysis (exon-by-exon) of the genes related to a particular clinical condition and MLPA analysis is needed to identify large deletions and duplications. In addition, no routine analysis of large genes with low mutation detection rates was performed due to cost-effective reasons. Massive parallel NGS technology has dramatically increased the throughput and reduced the cost per nucleotide sequenced, enabling the cost-effective sequencing of multiple genes in multiple patients. Taking into account that most IKD, including SRNS, are rare diseases with low prevalence, sequencing a disease-specific gene panel is not feasible to provide genetic diagnosis in a reasonable turn-over time. Based on this scenario, targeted sequencing of a global kidney panel including all the genes involved with cystic and glomerular IKD appears as a cost- and time-effective approach for genetic diagnosis of cystic and glomerular IKD. In terms of costs, our kidney panel approach is estimated to save 40% of costs per sample compared with sequential Sanger sequencing and MLPA analysis of candidate genes.

In this thesis, a kidney-disease gene panel including 132 genes involved in different glomerular and cystic kidney IKD was designed (Figure 27).

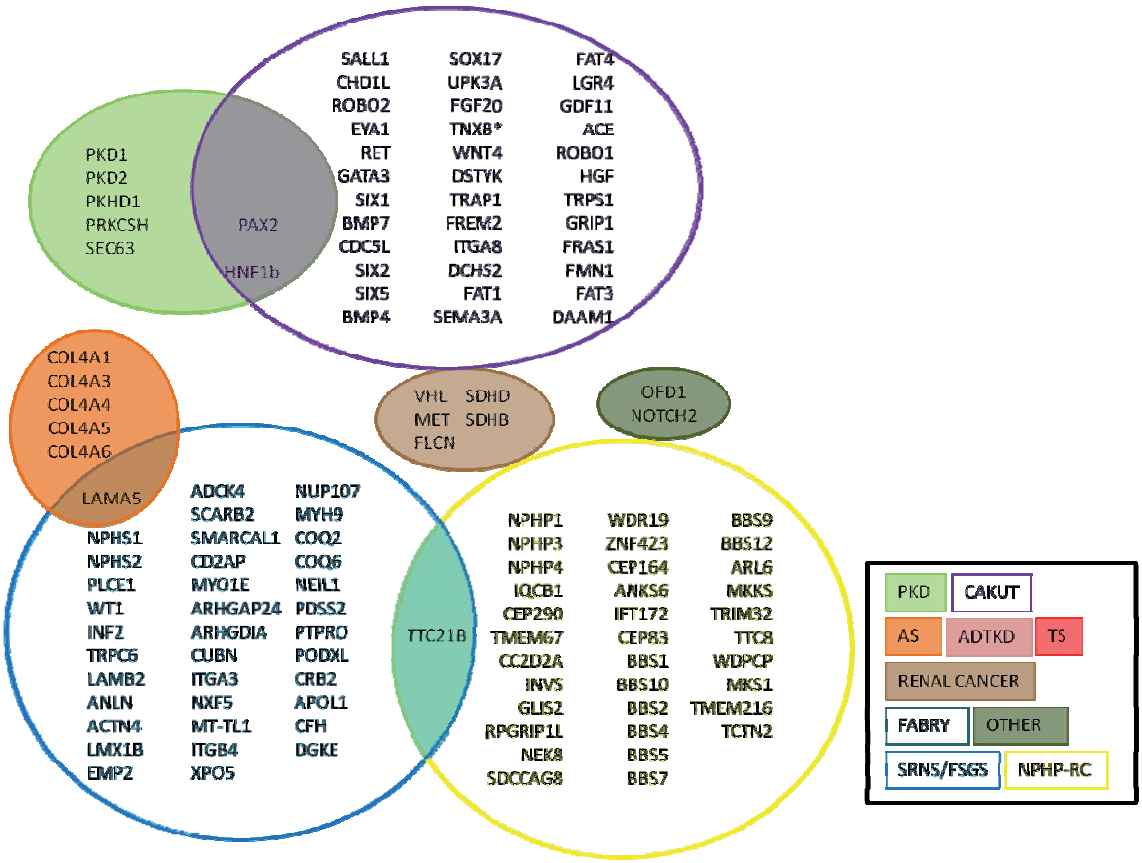

Figure 25: Schematic representation of the genes included in the kidney-disease gene panel according to the disease caused. ADTKD, autosomal dominant tubulointerstitial kidney disease; AS, alport syndrome; CAKUT, congenital anomalies of kidney and urinary tract; FSGS, focal segmental glomerulosclerosis; NPHP-RC, nephronophthisis-related ciliopathies; PKD, polycystic kidney disease; SRNS, steroid-resistant nephrotic syndrome; TS, tuberous sclerosis

Our kidney-disease gene panel demonstrated similar sensitivity than Sanger sequencing with the advantage of allowing a more comprehensive analysis of cystic and glomerular IKD patients. Specifically, our kidney-disease gene panel approach improves the genetic diagnosis of cystic and glomerular IKD by detecting 1) mutations in low-frequently mutated genes or genes that are differential diagnosis IKD and that were not routinely analyzed by Sanger sequencing; 2) mutations in a low proportion of reads, which in *de novo* cases is indicative of mosaicism; 3) structural variants, even those involving more than one exon; and 4) complex inheritance patterns or digenic inheritance, in whom hypomorphic alleles or additional mutations influence the disease phenotype.

1) <u>Mutations found in low-frequently mutated genes or differential diagnosis genes.</u> Mutations in the *NPHP3* and *CUBN* genes were found in a total of three patients. *NPHP3*

mutations were found in two fetuses with clinical features suggestive of ARPKD. Prenatal ultrasound in patient NPHP-001 revealed bilateral enlarged polycystic kidneys and anhydramnios. Patient NPHP3-002 resulted in a termination of pregnancy in the 21th week of gestation due to oligohydramnios and renal histology showed renal cystic dysplasia and a pancreatic hamartoma. Neonatal presentation of NPHP-RC can resemble ARPKD (Bergmann 2015). Thus, mutations in NPHP-RC genes may be responsible for the disease in some patients negative for *PKHD1* mutations. A homozygous mutation in *CUBN* gene was identified in a consanguineous patient with proteinuria. Mutations in the *CUBN* gene were initially described as causative of Imerslund-Gräsbeck syndrome, a form of congenital megaloblastic anemia due to vitamin B12 deficiency that in approximately half of patients is associated with proteinuria (Aminoff *et al.,* 1999). Nevertheless, a family with proteinuria without megaloblastic anemia has also been reported (Ovunc *et al.,* 2011). Our results add support to the role of *CUBN* mutations as a cause of proteinuria.

2) Mosaic mutations. Three patients with TS were found to carry mosaic mutations in a proportion of reads ranging from 9 to 15%. These patients presented with a mild disease without neurological involvement but they should be aware of the possibility of having severely affected offspring, such as patient TS-021. To detect mosaic mutations by massive parallel sequencing an extremely high depth of coverage is required. Confirmation of these mutations can be performed by Sanger sequencing if the proportion of mutated reads is approximately 10% or higher. In cases with less proportion of mutated reads detected allele-specific PCR o SNaPshot are required. Our three patients with mosaic mutations were all confirmed by Sanger sequencing.

3) Structural variants. Structural variants in collagen type IV genes were found in three patients, consisting of a duplication of *COL4A5* exons 38-41 (patient AS-258), a duplication of *COL4A3* exons 30-35 (patient AS-187), and a whole *COL4A3* deletion (patient SRNS-280). To the best of our knowledge, large deletions or duplications in *COL4A3* and *COL4A4* genes had only been reported in six patients (Morinière *et al.,* 2014), probably because screening for this type of genetic variation in these genes was rarely performed before the advent of NGS. Therefore, structural variants in *COL4A3* and *COL4A4* genes could account for a non-negligible proportion of patients.

4) Complex inheritance patterns. 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* mutation *in trans* with a *PKD1* hypomorphic allele is associated with early-onset disease; harboring homozygous or compound

heterozygous hypomorphic alleles may cause typical ADPKD or a severe ARPKD-like disease; and carrying one hypomorphic allele results in a mild cystic disease or may even be asymptomatic (Rossetti *et al.,* 2009; Vujic *et al.,* 2010). Complex inheritance patterns were found in three patients with renal cystic disease, two of them carrying two *PKD1* mutations and the remaining carrying combined heterozygous *PKD1* and *PKD2* mutations. All these patients presented a more severe phenotype than the one expected according to the mutated gene and the type of mutation (Cornec-Le Gall *et al.,* 2013), suggesting a contribution of both mutated alleles to the disease phenotype. In two of these three patients, no family members were available to determine if the mutations were inherited in *cis* or *trans* neither to know if they had renal cysts. As a result, the form of genetic transmission to their offspring could not be determined. In glomerular patients, heterozygous mutations in both *COL4A3* and *COL4A4* genes were detected in two families. Seven families with combined mutations in the *COL4A3* and *COL4A4* genes and a more severe phenotype than that expected for ADAS have recently been published (Mencarelli *et al.,* 2015).

The high throughput of massive parallel sequencing 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. On one hand, the more genes included in the panel, the more probability of identifying variants predicted to be pathogenic in unexpected genes. This fact is especially challenging in patients with no mutations found in the expected genes according to their phenotype. Large-scale sequencing projects have revealed the existence of many dozens of genetic variants predicted to severely disrupt protein-coding genes in the genomes of apparently healthy individuals, even in homozygous state (MacArthur & Tyler-Smith 2010). Thus, the assumption that variants that severely disrupt gene function are likely to be disease-causing must be cautiously taken and clinical information is essential to decide if the putative pathogenic variants play a role in the IKD analyzed. On the other hand, elucidating the contribution of additional variants in patients with putative complex inheritance patterns or digenic inheritance is also difficult. The pathogenicity of the additional variants needs to be evaluated considering their conservation among orthologues, population data and *in silico* predictors. In addition, it is essential the study of the segregation of these variants in as many family members as possible to elucidate their potential contribution to the disease as well as the form of genetic transmission, which will depend on whether the mutant alleles are on the same parental allele (*cis* configuration) or on different ones (*trans* configuration). Accurate clinical and genetic examination of these

families and experienced geneticists specialized in the diseases analyzed are needed for a correct genetic diagnosis and genetic counseling.

SRNS/FSGS: FROM GENETICS TO GENOMICS

To study SRNS and FSGS we used our kidney-disease gene panel but only the 26 glomerular genes were analyzed (Figure 26). The performance of our assay across the 26 genes was tested using the analysis of a validation cohort, consisting of 25 SRNS/FSGS patients with previously known mutations, and 5 previously genotyped controls. The identification of all previously known mutations in our validation cohort, as well as the high sensitivity and specificity obtained with the analysis of the previously genotyped controls, demonstrated the suitability of this approach for genetic diagnosis of SRNS/FSGS (Bullich *et al.,* 2015).

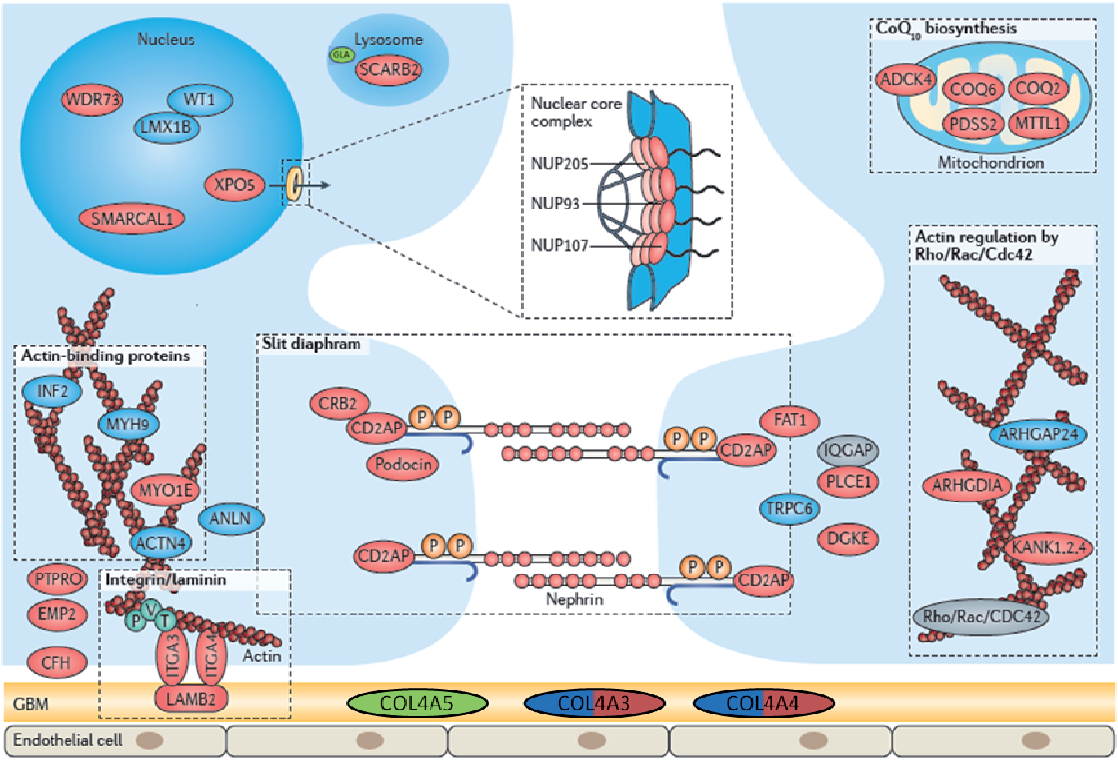

Figure 26. Proteins implicated in glomerular IKD grouped according to their structural activities or signaling pathways. The following proteins were not included in our glomerular panel because they had not been associated with glomerular IKD at the time of the study: WDR73, XPO5, NUP205, NUP93, NUP107, EMP2, ANLN, ITGA4, KANK1/2/4, ARHGDIA, ADCK4, and MTTL1. Our glomerular panel additionally included Cubilin and NEIL proteins. Red proteins indicate recessive forms of disease; blue proteins indicate dominant forms of disease; green proteins indicate X-linked forms of disease and grey proteins have not associated with glomerular IKD (Modified from Vivante & Hildebrandt 2016).

Mutation analysis of our discovery cohort revealed disease-causing mutations in 36% (9 of 25) of patients (Bullich *et al.,* 2015). Similarly, two studies using a targeted sequencing of 24 and 21 SRNS-related genes, identified disease-causing mutations in 30.6% (11 of 36) and 33%

(16 of 48) of their discovery patients, respectively (McCarthy *et al.,* 2013; Lovric *et al.,* 2014). Studies in larger international cohorts of patients also led to similar mutation detection rates. Sadowski *et al.* analyzed 27 SRNS-causing genes and identified single-gene cause in 29.5% (526 of 1783) of families that manifested before 25 years of age (Sadowski *et al.,* 2014). Trautmann *et al.* identified a genetic cause in 23.6% (277/1174) of the patients screened by combining Sanger sequencing of *NPHS2* and *WT1* and NGS of 31 podocyte genes (Trautmann *et al.,* 2015). In all of these studies, the percentage of patients with mutations decreases as the age at disease onset increases (Table 4).

		Age at disease onset					
Reference	Patients identified mutation (%)	Congenital onset	Infantile onset	Early childhood onset	Late childhood onset	Adolescence onset	Adult onset
Bullich *et al.*, 2015	9/25 (36)	5/5 (100)	2/9 (22.2)		0/3 (0)	0/0 (0)	2/8 (25)
Sadowski *et al.*, 2015	526/1783 (29.5)	163/235 (69.4)	81/163 (49.7)	177/700 (25.3)	56/315 (17.8)	19/176 (10.8)	6/28 (21.4)
Trautmann *et al.*, 2015	277/1174 (23.6)	55/83 (66.3)	31/87 (35.6)	119/546 (50.9)	40/256 (22.7()	24/149 (16.1)	

Table 4. Patients with causative mutation detected per age group in different studies. Onset was classified as follows: congenital, 0-3 months; infantile 4-12 months; early-childhood, 1-6 years; late-childhood, 7-12 years; adolescence, 13-18 years; adult, >18. Lovric *et al.* was not considered as the patients in this study were also included in the study by Sadowski *et al.* in which the authors considered a larger cohort of patients

The large proportions of patients with no pathogenic mutation identified suggest that genes as yet non-identified might be responsible for the disease in some patients, especially in very early-onset patients and familial cases. Also, some patients with no pathogenic mutation detected present disease recurrence after kidney transplantation, indicating that immunological factors are likely to be the cause of the disease.

Our discovery cohort included four patients in whom only one mutation in *NPHS1* (n=2) or *NPHS2* (n=2) was identified. A large insertion/deletion or deep intronic splicing mutation as a second pathogenic mutation was expected. However, only variants in non-canonical splice sites were found in three of them (Bullich *et al.,* 2015).

Tri-allelic inheritance of *NPHS1* and *NPHS2* mutation was suggested in a few families with SRNS/FSGS (Koziell *et al.,* 2002; Lowik *et al.,* 2008), but controversy exists about the contribution of these additional recessive variants to the disease phenotype (Schultheiss *et al.,* 2004). The p.R229Q variant in *NPHS2* gene has been suggested to contribute to proteinuria and ESRD in collagen type IV (α3α4) nephropathy (Tonna *et al.,* 2008; Voskarides *et al.,* 2012). Additional heterozygous variants in a recessive gene were recently identified in 22% of SRNS patients. However, the contribution of these variants to the disease was not proved (Weber *et al.,* 2016). Of note, a preconception carrier screen for 448 severe recessive childhood diseases

revealed an average genomic carrier burden for severe recessive mutations of 2.8 (ranging from 0 to 7) (Bell *et al.,* 2011). Taken together, it might be speculated that the contribution of heterozygous additional variants in recessive genes to the disease phenotype is rather limited.

On the contrary, our results showed that additional heterozygous variants in a dominant gene may contribute to increase disease severity. We identified three patients carrying mutations in an SRNS/FSGS gene together with a heterozygous mutation *COL4A3*. The clinical phenotype of these three patients stands out for the co-existence of NS and microhematuria at presentation. Two of them were familial cases that presented a more severe phenotype than their family members with mutations in only one gene (Bullich *et al.,* 2015). Most of the SRNS/FSGS-related genes encode podocyte proteins whereas COL4A3 protein is a component of the GBM. The GFB integrates the functions of its constituent layers with dynamically intertwined roles that harmonize together into one functionally elegant ensemble (Scott & Quaggin 2015). Therefore, it is plausible that patients with mutations affecting podocytes and GBM proteins have a more severe phenotype than patients carrying mutations in "only" one podocyte proteins.

Similarly, the identification of heterozygous mutations in *COL4A3* or *COL4A4* genes as causative of adult-onset FSGS has also broadened the genetic spectrum associated with this pathological diagnosis (Voskarides *et al.,* 2007; Malone *et al.,* 2014). However, until the advent of NGS technologies, the only genes contemplated for genetic diagnosis of adult-onset FSGS were *NPHS2*, *INF2, TRPC6*, and/or *ACTN4* (Santín, Bullich, *et al.,* 2011; Benoit *et al.,* 2010).

Based on our results and the revision of the literature, the genotypic and phenotypic spectrum associated with glomerular IKD has broadened (Figure 27). In children, SRNS and AS are usually well-defined entities because the phenotype is almost exclusively determined by the causative gene. Clinical manifestations of SRNS are nephrotic range proteinuria, edemas, hipoproteinemia and hyperlipidemia whereas AS is characterized by hematuria, proteinuria (usually not in the nephrotic range), progressive renal failure, and, often, high-tone sensorineural hearing loss and specific ocular lesions. In adult-onset patients, both diseases converge in a much more similar phenotype due to the reduced tightness of genotype-phenotype correlations. FSGS is usually found in both diseases, moreover in many adults proteinuria in idiopathic FSGS is below the nephrotic range as it is true for AS. Thus, SRNS and AS genes may be the cause of the renal disease in adult patients with proteinuria. The coexistence with hematuria would point more to AS and its absence to primary FSGS.

However, as these phenotypes frequently overlap, the use of a renal panel including all genes located in the GFB is extremely welcome.

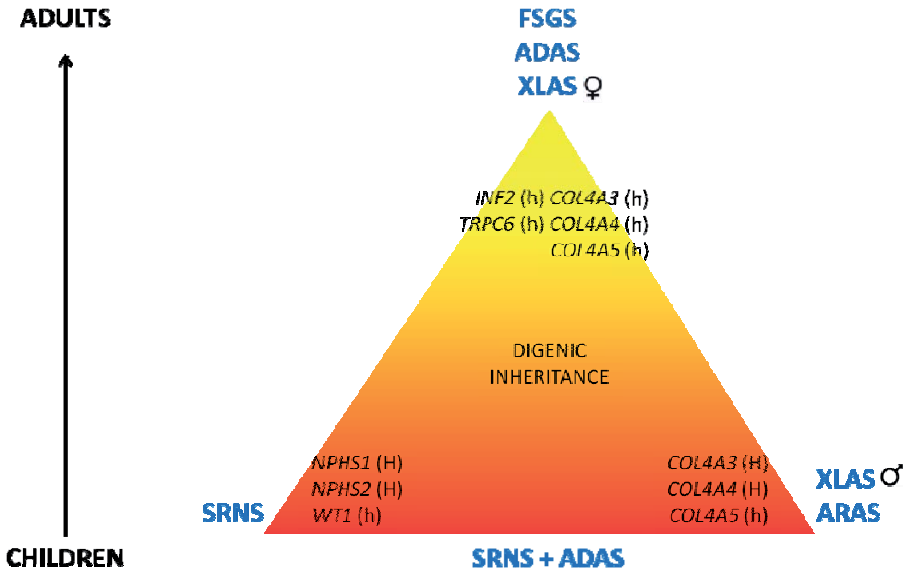

Figure 27. Schematic representation of the genotypic and phenotypic spectrum of glomerular IKD. The disease severity ranges from severe (orange) to mild (yellow). Abbreviations: ADAS, autosomal dominant alport syndrome; ARAS, autosomal recessive alport syndrome; FSGS, focal segmental glomerulosclerosis; H, homozygous; h, heterozygous; SRNS, steroid-resistant nephrotic syndrome; XLAS, X-linked alport syndrome

TTC21B GENE: CAUSATIVE AND MODIFYING ROLE

Mutations in the ciliary gene *TTC21B* were described as causative of a broad range of ciliopathies (Davis *et al.,* 2011; McInerney-Leo *et al.,* 2014; Otto *et al.,* 2011). Patients with at least one truncating or splice site mutation exhibit syndromic ciliopathies, whereas all patients carrying the homozygous p.P209L had isolated renal disease. The homozygous p.P209L mutation was initially identified in three patients with isolated nephronphthisis (Davis *et al.,* 2011) but it has been recently found in seven families with FSGS (Huynh Cong *et al.,* 2014). Re-examination of the renal biopsies of these 10 patients carrying the homozygous p.P209L mutation revealed both FSGS and tubulointerstitial lesions characteristic of nephronophthisis in all of them. This finding defined a new entity combining glomerular and tubulointerstitial damages (Huynh Cong *et al.,* 2014).

In this thesis, the causative role of the homozygous p.P209L *TTC21B* mutation in patients with both FSGS and tubulointerstitial lesions was confirmed. Moreover, a family with two siblings presenting FSGS that carried compound heterozygous mutations in *TTC21B*: p.P209L and p.H426D was also identified, demonstrating that missense mutations different from the

homozygous p.P209L can also cause isolated renal disease. Interestingly, these siblings had an earlier age at onset of proteinuria than the homozygous p.P209L patients, suggesting that the p.H426D mutation might be more deleterious than the p.P209L mutation (Bullich *et al.,* 2016).

Heterozygous *TTC21B* mutations have been suggested to contribute as putative second-site modifiers in ciliopathy patients based on the significant enrichment of *TTC21B* pathogenic variants in these patients compared to controls (Davis *et al.,* 2011). In this thesis, no significant enrichment for the total set of rare *TTC21B* pathogenic variants was found in cystic and glomerular patients compared to controls (Bullich *et al.,* 2016). The differences in the renal diseases considered, the smaller size of our case cohort and the fact that Davis *et al* sequenced their control group while we obtained control data from 1000 Genomes, might explain the differences between the two studies.

Five patients carrying a heterozygous *TTC21B* variant predicted to be deleterious in addition to the disease-causing mutations were identified in our cohort, four with a glomerular disease and one with a cystic disease. These *TTC21B* variants were significantly more frequent in our renal patients compared to controls. Clinical examination of these patients revealed that two of these five patients had a more severe phenotype that expected for the disease-causing mutation. Patient 61 with ADPKD carried a missense *PKD1* mutation and a *TTC21B* heterozygous variant. He presented with CKD stage IV at 36 years whereas patients with *PKD1* non-truncating mutations have a median age at ESRD of 68 years (Cornec-Le Gall *et al.,* 2013). Patient 82 carried a *COL4A3* mutation in heterozygous state together with a frameshift mutation in *TTC21B*. She presented with microhematuria, full-blown NS, and high blood pressure. Her renal biopsy showed FSGS with tubular atrophy and interstitial fibrosis. She reached ESRD at 32 years, while only around 29% of ADAs patients progress to ESRD at a mean age of 56 (Fallerini *et al.,* 2014). The remaining three patients carrying an additional *TTC21B* deleterious variant had childhood-onset recessive diseases with short-term follow-up. Thus, the contribution of the *TTC21B* variant was more difficult to assess. Our results suggest that deleterious *TTC21B* alleles likely aggravate the phenotype in patients with glomerular and cystic diseases, adding support to their proposed modifying role (Bullich *et al.,* 2016).

CONCLUSIONS

1- *HLA-DQA1* (rs2187668) and *PLA2R1* (rs4664308) are associated with idiopathic membranous nephropathy (IMN) development in the Spanish population. The combination of the risk alleles for both SNPs results in an increased risk of IMN.

2- CNVs in *FCGR3A* and *FCGR3B* genes are not associated with susceptibility to IMN in the Spanish population.

3- *HLA-DQA1* and *PLA2R1* genotype combination adjusted for baseline proteinuria predicts the response to immunosuppressive therapy and renal function decline.

4- Targeted sequencing of a glomerular disease gene panel is feasible and robust for genetic diagnosis of SRNS/FSGS.

5- Patients carrying mutations in an SRNS/FSGS gene and also in *COL4A3* have increased disease severity. Mutations in different genes that converge in the glomerular filtration barrier explain some of the phenotypic variability in SRNS/FSGS.

6- The *TTC21B* gene should be considered for the genetic diagnosis of families with FSGS and tubulointerstitial lesions. The homozygous p.P209L mutation is not the only causative mutation of this nephropathy.

7- Heterozygous deleterious *TTC21B* variants may aggravate the phenotype of patients with glomerular and cystic kidney inherited kidney diseases.

8- Massive parallel sequencing of the kidney-disease gene panel is an efficient and cost-effective tool for genetic diagnosis of cystic and glomerular inherited kidney diseases.

9- The kidney-disease gene panel approach allows for a more comprehensive characterization of patients enabling the detection of very rarely mutated genes, complex inheritance patterns and coinheritance of mutations in genes responsible for different inherited kidney diseases.

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ANNEXES

Trujillano D, **Bullich G**, Ossowski S, Ballarín J, Torra R, Estivill X, Ars E. **Diagnosis of autosomal dominant polycystic kidney disease using efficient *PKD1* and *PKD2* targeted next-generation sequencing.** Mol Genet Genomic Med 2014, 2(5):412-421.

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ORIGINAL ARTICLE

Diagnosis of autosomal dominant polycystic kidney disease using efficient *PKD1* and *PKD2* targeted next-generation sequencing

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Abstract

Molecular diagnostics of autosomal dominant polycystic kidney disease (ADPKD) relies on mutation screening of *PKD1* and *PKD2*, which is complicated by extensive allelic heterogeneity and the presence of six highly homologous sequences of *PKD1*. To date, specific sequencing of *PKD1* requires laborious long-range amplifications. The high cost and long turnaround time of *PKD1* and *PKD2* mutation analysis using conventional techniques limits its widespread application in clinical settings. We performed targeted next-generation sequencing (NGS) of *PKD1* and *PKD2*. Pooled barcoded DNA patient libraries were enriched by in-solution hybridization with *PKD1* and *PKD2* capture probes. Bioinformatics analysis was performed using an in-house developed pipeline. We validated the assay in a cohort of 36 patients with previously known *PKD1* and *PKD2* mutations and five control individuals. Then, we used the same assay and bioinformatics analysis in a discovery cohort of 12 uncharacterized patients. We detected 35 out of 36 known definitely, highly likely, and likely pathogenic mutations in the validation cohort, including two large deletions. In the discovery cohort, we detected 11 different pathogenic mutations in 10 out of 12 patients. This study demonstrates that laborious long-range PCRs of the repeated *PKD1* region can be avoided by in-solution enrichment of *PKD1* and *PKD2* and NGS. This strategy significantly reduces the cost and time for simultaneous *PKD1* and *PKD2* sequence analysis, facilitating routine genetic diagnostics of ADPKD.

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD; OMIM IDs: 173900; 613095) is the most common inherited kidney disease, with an incidence of 1 in 400–1000 (Iglesias et al. 1983; Dalgaard and Norby 1989). ADPKD is caused by mutations in *PKD1* (16p13.3; OMIM ID: 601313) in approximately 85% of the cases (The European Polycystic Kidney Disease Consortium 1994), and in *PKD2* (4q21; OMIM ID: 173910) in the remaining 15% (Mochizuki et al. 1996). ADPKD is characterized by the development and progressive enlargement of cysts in the kidneys and other organs, eventually leading to end-stage renal disease (ESRD). The ADPKD phenotype displays a significant variability that is greatly influenced by the affected gene. Thus, *PKD1* patients have a median age at ESRD of 58 years compared to 79 years for *PKD2* mutated patients (Cornec-Le Gall et al. 2013).

Diagnosis of ADPKD is mainly performed by renal imaging such as ultrasonography, computed tomography, or magnetic nuclear resonance (Pei et al. 2009). However, molecular diagnostics is necessary in several situations: (1) when a definite diagnosis is required in young individuals, such as a potential living related donor in an affected family with equivocal imaging data; (2) in patients with a negative family history of ADPKD, because of potential phenotypic overlap with several other kidney cystic diseases; (3) in families affected by early-onset polycystic kidney disease, since in this cases hypomorphic alleles and/or oligogenic inheritance can be involved (Rossetti et al. 2009; Bergmann et al. 2011; Harris and Hopp 2013); and (4) in patients requesting genetic counseling, especially in couples wishing a preimplantation genetic diagnosis (Harris and Rossetti 2010).

Approximately 70% of the 5′ genomic region of the *PKD1* gene (exons 1–33) is duplicated six times on chromosome 16p within six pseudogenes (*PKD1P1 to PKD1P6*), which share a 97.7% sequence identity with the genuine gene (Bogdanova et al. 2001; Rossetti et al. 2012). This, together with a high GC content, the presence of many missense variants, the absence of mutation hot spots, and the high allelic heterogeneity of ADPKD, makes the molecular diagnostics of ADPKD challenging. In addition, most mutations are private variants, with a total of 1272 pathogenic *PKD1* and 202 pathogenic *PKD2* mutations reported to date (March 2014, ADPKD Database [PKDB], http://pkdb.mayo.edu). Thus, genetic diagnosis by conventional techniques of a new ADPKD family requires long-range polymerase chain reaction (LR-PCR) of the repeated region of *PKD1* followed by nested PCRs (Rossetti et al. 2002), combined with Sanger sequencing of all 46 *PKD1* and 15 *PKD2* exons. When pathogenic mutations are not identified by Sanger sequencing, multiplex ligation-dependent probe amplification (MLPA) analysis is also performed to identify potential insertions and deletions.

Therefore, there is a demand for more simple and cost-effective molecular approaches that could be used for routine diagnosis, especially now with the coming specific therapies that will require differential genetic diagnosis (Torres and Harris 2006). To address these challenges, we have developed and validated an assay that couples genome partitioning and next-generation sequencing (NGS), to comprehensively perform in one-step mutation screening in *PKD1* and *PKD2*, as an alternative to cumbersome conventional genetic testing methods.

Material and Methods

Subjects

High-quality genomic DNA from 53 unrelated patients was obtained from blood lymphocytes, using standard protocols. The validation cohort included 36 ADPKD patients and five control individuals that had previously undergone conventional genetic diagnosis by Sanger sequencing of all *PKD1* and *PKD2* exons and, if negative, MLPA was also applied. The discovery cohort consisted of 12 ADPKD consecutive patients received for genetic diagnosis for which no mutations were known. ADPKD diagnosis was based on standard clinical and imaging criteria. Blood samples were obtained from other family members if they were available. All samples were codified and bioinformatics mutation analysis was blindly performed. Signed informed consent was obtained for all participants. This study was approved by the institutional review board.

Capture and multiplexed sequencing of the *PKD1* and *PKD2* genes

To carry out DNA capture, we designed a custom NimbleGen SeqCap EZ Choice Library (Roche, Inc., Madison, WI) to target the complete genomic sequence of the *PKD1* and *PKD2* genes, and 1 kb of genomic sequence flanking at the 5′ and 3′ ends of each gene, accounting for 121,322 bp. Our design also included probes to target additional genes related to other human inherited diseases, for a total of 2.1 Mb of captured DNA after removal of repetitive sequences. DNA probes were selected using the most stringent settings for probe design (uniqueness tested by Sequence Search and Alignment by Hashing Algorithm [SSAHA]) (Ning et al. 2001). However, in order 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. The Browser Extensible Data file of captured regions is available on request to the authors.

Libraries were prepared with the TruSeq DNA Sample Preparation Kits (Illumina, Inc., San Diego, CA). Genomic capture from pooled libraries was carried out using NimbleGen SeqCap EZ Library (Roche, Inc.) following User's Guide v3.0 instructions, as previously described (Trujillano et al. 2013). The libraries of the patients of the validation cohort and the five controls were prepared and sequenced together with seven samples of other diseases using the same capture design and enrichment protocol in two pools of 24 samples, for a total of 48 samples multiplexed in two HiSeq 2000 (Illumina, Inc.) lanes to generate 2 × 100 bp paired-end reads. The 12 patients of the discovery cohort were enriched in a single capture reaction and were sequenced in a Miseq (Illumina, Inc.) run to generate 2 × 250 bp paired-end reads.

Bioinformatics analysis and mutation identification and classification

The resulting fastq files were analyzed with an in-house developed pipeline previously described (Trujillano et al. 2013). All the bioinformatics tools used in this study were run using default settings unless stated otherwise. For the patients included in this study, only the sequencing data produced for *PKD1* and *PKD2* were analyzed, as stated in the signed informed consent. The reference sequences used were NM_001009944.2 for *PKD1* and NM_000297.2 for *PKD2*. In order to identify pathogenic mutations that could cause ADPKD, we applied the following cascade of filtering steps (Walsh et al. 2010):

1 We required all candidate variants on both sequenced DNA strands and to account for ≥20% of total reads at that site in order to filter out spurious variant calls caused by misaligned reads in the duplicated region of *PKD1*.
2 Common polymorphisms (≥5% in the general population) were discarded by comparison with dbSNP 137, the 1000G, the Exome Variant Server (http://evs.gs.washington.edu), and an in-house exome variant database to filter out both common benign variants and recurrent artifact variant calls, especially in the duplicated *PKD1* regions. However, since these databases also contain known disease-associated mutations, all detected variants were compared to gene mutation databases (The Human Gene Mutation Database [HGMD], www.hgmd.cf.ac.uk and ADPKD Database [PKDB], http://pkdb.mayo.edu).
3 Mutations that could give rise to premature truncated proteins, that is, stop mutations, exonic deletions/insertions, and large genomic rearrangements were classified as definitely pathogenic.
4 Missense and noncanonical splicing variants were considered a priori Unclassified Sequence Variants (UCV) and their potential pathogenicity was evaluated using an in silico scoring system developed for *PKD1* and *PKD2* genes as previously described (Rossetti et al. 2007). This scoring system takes into consideration a number of in silico predictors (Grantham 1974; Tavtigian et al. 2006; Rossetti et al. 2007) and population data. We scored each of these factors, the sum of which resulted in an overall Variant Score (VS). The UCV were classified into four groups: highly likely pathogenic (VS ≥ 11);

likely pathogenic ($5 \leq VS \leq 10$), indeterminate ($0 \leq VS \leq 4$), and highly likely neutral ($VS \leq -1$) (Rossetti et al. 2007).

We considered to be pathogenic mutations those sequence variants predicted to result in a truncated protein (classified as definitely pathogenic) and those not found in healthy controls, that segregated with the disease in families and expected to severely alter the protein sequence using in silico predictors (classified as highly likely pathogenic and likely pathogenic variants).

If no pathogenic mutations were identified, the bioinformatics pipeline automatically reported the target sequences that presented low or inexistent sequence coverage. These regions were screened by Sanger sequencing since they were more likely to contain the pathogenic variants missed by our NGS approach. Validation of newly identified single-nucleotide variants (SNVs) was performed by Sanger sequencing.

Results

PKD1 and *PKD2* enrichment

Eighty-one percent of the *PKD1*- and *PKD2*-targeted bases could be covered with capture baits for a final targeted region of 98,524 bp divided into 99 individual regions, with lengths ranging from 65 to 6,493 bp (average of 995 bp) (Table S1). Noteworthy, 100% of all coding sequences, that is, the complete 46 and 15 exons of *PKD1* and *PKD2*, respectively, were covered by capture baits. The target regions that precluded bait tilling correspond only to intronic and intergenic sequences.

Sequencing statistics

In the validation cohort, an evenly distributed mean depth of coverage of 331X and 481X for *PKD1* and *PKD2* was achieved, respectively, on average across samples (Table 1). We achieved a sequencing depth of 289X for the 46 exons of *PKD1* and 453X for the 15 exons of *PKD2*, on average across samples (Table S2). Ninety-five percent of the coding base pairs of *PKD1* and 94% of *PKD2* were covered by more than 20 reads, which is enough for an accurate detection of known and novel mutations. Only exons 1 and 42 of *PKD1* and exon 1 of *PKD2* were not captured and sequenced at an adequate read depth (Fig. 1).

Due to the lower throughput of the MiSeq sequencer, the average coverage achieved in the discovery cohort was of 81X and 174X for *PKD1* and *PKD2*, respectively, across the 12 samples (Table 1). For a comprehensive summary of the obtained sequencing results, see also Tables S3, S4.

Detection of *PKD1* and *PKD2* mutations in the validation cohort

For the validation cohort we selected samples with as many different types of *PKD1* and *PKD2* mutations as possible, including SNVs, short insertions and deletions (InDels), and large structural variants (SVs). We identified 35 out of 36 previously known different pathogenic mutations (30 in *PKD1* and five in *PKD2*) in their correct heterozygous state (Table 2). These results would have led to a diagnostic rate of 97.2%. Noteworthy, 25 (70%) of these mutations were spread along different exons within the segmentally duplicated regions of the *PKD1* gene, highlighting the robustness of our approach even for

Table 1. Average sequencing quality control and coverage statistics of *PKD1* and *PKD2* in the validation and discovery cohorts.

	Validation		Discovery	
Cohort	Average	SD	Average	SD
QC-passed reads	14452006.67	2252761.13	1303016.25	293339.48
Mapped	14328976.12	2236282.41	1002567.63	269009.02
Properly paired	14140971.70	2203337.48	780154.25	265250.15
PKD1 mean coverage (X)	331.14	89.20	80.60	13.60
% *PKD1* target bases covered = 0X	1.98	0.35	3.70	0.39
% *PKD1* target bases covered ≥ 1X	98.02	0.35	98.15	0.20
% *PKD1* target bases covered ≥ 20X	95.54	1.98	86.69	2.59
% *PKD1* target bases covered ≥ 50X	92.40	4.13	65.03	4.76
% *PKD1* target bases covered ≥ 100X	84.78	6.84	52.01	1.65
PKD2 mean coverage (X)	480.73	87.98	174.22	28.72
% *PKD2* target bases covered = 0X	0.36	0.11	0.90	0.20
% *PKD2* target bases covered ≥ 1X	99.64	0.11	99.55	0.10
% *PKD2* target bases covered ≥ 20X	99.19	0.20	98.75	0.31
% *PKD2* target bases covered ≥ 50X	98.68	0.37	92.74	3.46
% *PKD2* target bases covered ≥ 100X	97.10	2.22	67.51	8.70

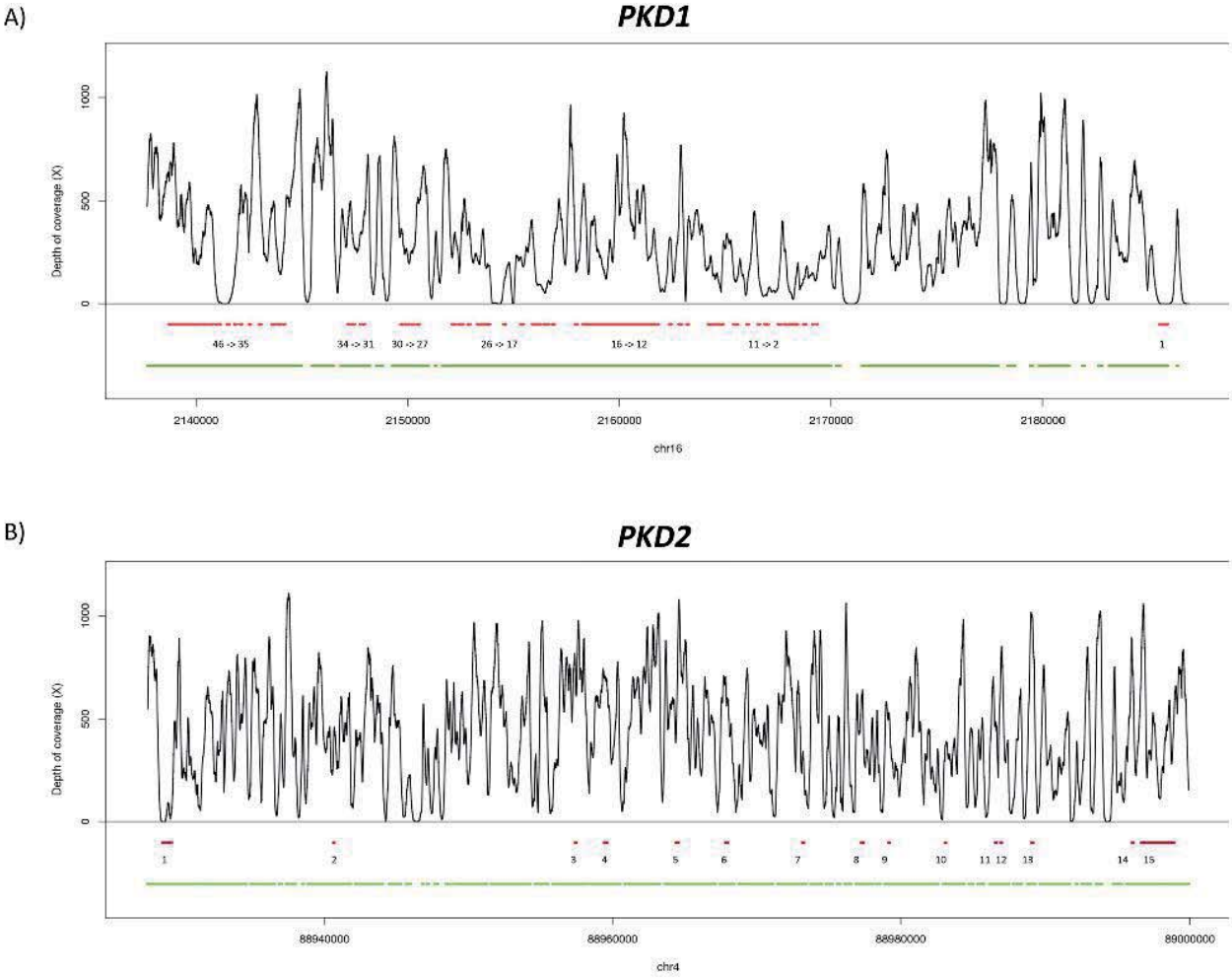

Figure 1. Representation of the average depth of coverage of *PKD1* (A) and *PKD2* (B) in the validation cohort. Red lines and the numbers underneath represent the exons of the genes. Green lines represent the regions tilled by capture baits.

genes with highly homologous pseudogenes. In addition, two previously known large deletions were correctly detected. Concretely, patient 03-106-P6 presented *PKD1* g.2154344-2186386del (Fig. 2A), and patient 11-571-P2 presented *PKD2* g.88952828-89050618del (Fig. 2B). For the unique patient with a previously known mutation not identified by our NGS assay, 03-393-P3, manual inspection of the sequence alignment files revealed that the missing p.(Met1fs) was localized in a region of exon 1 of *PKD1* for which no NGS reads were available due to problems with the genomic capture.

We included in this study five control individuals without personal or family history of ADPKD to determine the clinical specificity of our assay. These controls had been previously genotyped with a HumanOmni 2.5-8 BeadChip (Illumina, Inc.) and were also used to determine the analytic sensitivity of our assay to detect heterozygous and homozygous SNVs. Genotype data were available for a total of 80 and 269 sites within the targeted regions of *PKD1* and *PKD2*, respectively. Sensitivity was of 100% both for *PKD1* (20/20) and *PKD2* (103/103). Analytic specificity was 100% both for *PKD1* (60/60) and *PKD2* (166/166) (Table S5). Of note, no spurious pathogenic calls were detected in either the control individuals or the validation cohort.

Identification of *PKD1* and *PKD2* mutations in the discovery cohort

We detected pathogenic mutations in 10 out of 12 patients carrying a total of 11 different pathogenic mutations (10 in *PKD1* and one in *PKD2*), which lead to a diagnostic rate of 83.3%. All variants were confirmed by Sanger sequencing (Table 3). Interestingly, one patient (12–444) harbored one definitively pathogenic mutation in *PKD2* and one highly likely pathogenic mutation in

Table 2. ADPKD mutations in *PKD1* and *PKD2* identified in the 36 samples of the validation cohort.

Sample	Gene	Duplicated region	cDNA change	Protein change	PKDB	# Patients	Classification	Ref counts	Variants counts
03-106-P6	*PKD1*	Yes	c.1-?_8161+?del	p.(Met1fs)	Absent	0	Definitely pathogenic	–	–
12-331-P1	*PKD1*	Yes	c.566C>G	p.(Ser189*)	Present	1	Definitely pathogenic	188	62
12-382-P1	*PKD1*	Yes	c.736_737del	p.(Ser246fs)	Absent	0	Definitely pathogenic	19	14
04-016-P6	*PKD1*	Yes	c.1831C>T	p.(Arg611Trp)	Present	1	Likely pathogenic	25	13
12-235-P1	*PKD1*	Yes	c.2329C>T	p.(Gln777*)	Absent	0	Definitely pathogenic	59	37
12-366-P1	*PKD1*	Yes	c.2478delC	p.(Ile827 fs)	Absent	0	Definitely pathogenic	114	83
12-010-P1	*PKD1*	Yes	c.4888C>T	p.(Gln1630*)	Present	1	Definitely pathogenic	136	114
02-010-P6	*PKD1*	Yes	c.6583_6589del7	p.(Cys2195fs)	Present	1	Definitely pathogenic	74	79
10-326-P3	*PKD1*	Yes	c.6778_6780delATT	p.(Ile2260del)	Present	1	Highly likely Pathogenic	190	148
11-220-P2	*PKD1*	Yes	c.6221delA	p.(Asn2074fs)	Absent	0	Definitely Pathogenic	123	113
11-247-P7	*PKD1*	Yes	c.6384C>A	p.(Asn2128Lys)	Absent	0	Highly likely pathogenic	181	131
12-161-P1	*PKD1*	Yes	c.6586C>T	p.(Gln2196*)	Present	1	Definitely pathogenic	86	50
11-517-P1	*PKD1*	Yes	c.6736C>T	p.(Gln2246*)	Present	1	Definitely pathogenic	185	124
11-525-P2	*PKD1*	Yes	c.6827T>C	p.(Leu2276Pro)	Absent	0	Highly likely pathogenic	280	245
10-388-P3	*PKD1*	Yes	c.8161+1G>C	p.(?)	Absent	0	Definitely pathogenic	21	25
11-468-P1	*PKD1*	Yes	c.8251C>T	p.(Gln2751*)	Absent	0	Definitely Pathogenic	92	92
12-363-P1	*PKD1*	Yes	c.8285delT	p.(Ile2762fs)	Absent	0	Definitely Pathogenic	120	89
10-463-P3	*PKD1*	Yes	c.8311G>A	p.(Glu2771Lys)	Present	18	Highly likely pathogenic	58	63
11-457-P2	*PKD1*	Yes	c.8858A>G	p.(Asn2953Ser)	Absent	0	Highly likely pathogenic	236	191
11-287-P2	*PKD1*	Yes	c.9240_9241delAT	p.(Ala3082fs)	Present	3	Definitely pathogenic	164	80
10-193-P3	*PKD1*	Yes	c.9412G>A	p.(Val3138Met)	Present	2	Likely Pathogenic	208	188
11-595-P2	*PKD1*	Yes	c.9455_9456insC	p.(Arg3152fs)	Absent	0	Definitely pathogenic	283	184
07-172-P5	*PKD1*	Yes	c.9889G>A	p.(Val3297Met)	Absent	0	Likely pathogenic	130	114
09-403-P3	*PKD1*	Yes	c.10170+25_+45del19	p.(Gln3390fs)	Present	2	Highly likely pathogenic	96	26
10-182-P3	*PKD1*	–	c.11017-10C>A	p.(Arg3672fs)	Present	7	Highly likely pathogenic	109	83
12-144-P1	*PKD1*	–	c.10847C>A	p.(Ser3616*)	Absent	0	Definitely Pathogenic	224	177
10-353-P3	*PKD1*	–	c.11359_11360del	p.(Pro3788fs)	Absent	0	Definitely pathogenic	292	203
11-256-P2	*PKD1*	–	c.11471G>T	p.(Gly3824Val)	Absent	0	Likely pathogenic	85	63
11-168-P8	*PKD1*	–	c.12004-2A>G	p.(?)	Absent	0	Definitely pathogenic	143	113
09-446-P3	*PKD1*	–	c.12031C>T	p.(Gln4011*)	Present	4	Definitely pathogenic	106	85
11-133-P8	*PKD2*	–	c.224delC	p.(Pro75fs)	Absent	0	Definitely pathogenic	41	25
11-008-P3	*PKD2*	–	c.637C>T	p.(Arg213*)	Absent	0	Definitely pathogenic	198	172
11-170-P2	*PKD2*	–	c.965G>A	p.(Arg322Gln)	Present	4	Highly likely pathogenic	396	330
12-149-P1	*PKD2*	–	c.2050_2053del4	p.(Tyr684fs)	Present	1	Definitely pathogenic	212	181
11-571-P2	*PKD2*	–	c.709-?_2907+?del	p.(Leu237_Val968del)	Absent	0	Definitely pathogenic	–	–
09-393-P3	–	–	–	–	–		–	–	–

#Patients in previous studies, NM_001009944.2 for *PKD1* and NM_000297.2 for *PKD2*

PKD1, presenting a more severe phenotype compared to the rest of the family. For the two samples in which no pathogenic variants were identified with our NGS assay, the bioinformatics pipeline proposed a list of candidate regions with low sequence coverage that were screened by Sanger sequencing and the two causal mutations were identified. Then, by manually inspecting the alignment files of the NGS reads, we realized that we had lost p.(Val2768Met) in patient 13-102 and p.(Arg4021fs) in patient 07-335 because their locations were in poorly covered areas of *PKD1* and the variant calling and filtering algorithms had discarded them as potential false positives calls. Noteworthy, no spurious pathogenic calls were reported in any of the samples of the discovery cohort.

Discussion

It has been suggested that the impact that NGS technologies will have on clinical genetics during the upcoming years will be comparable to the introduction of X-rays to medicine many decades ago (Hennekam and Biesecker 2012). After the tremendous impact of NGS technologies in the discovery of disease-causing genes during the last 4 years, we are witnessing the introduction of these technologies for diagnostic applications, with the aim of rapidly revolutionize the field of genetic diagnostics, making it much more cost- and time-effective, advance accuracy, and point to unsuspected yet treatable conditions. The purpose of this study was to develop a cost-effective

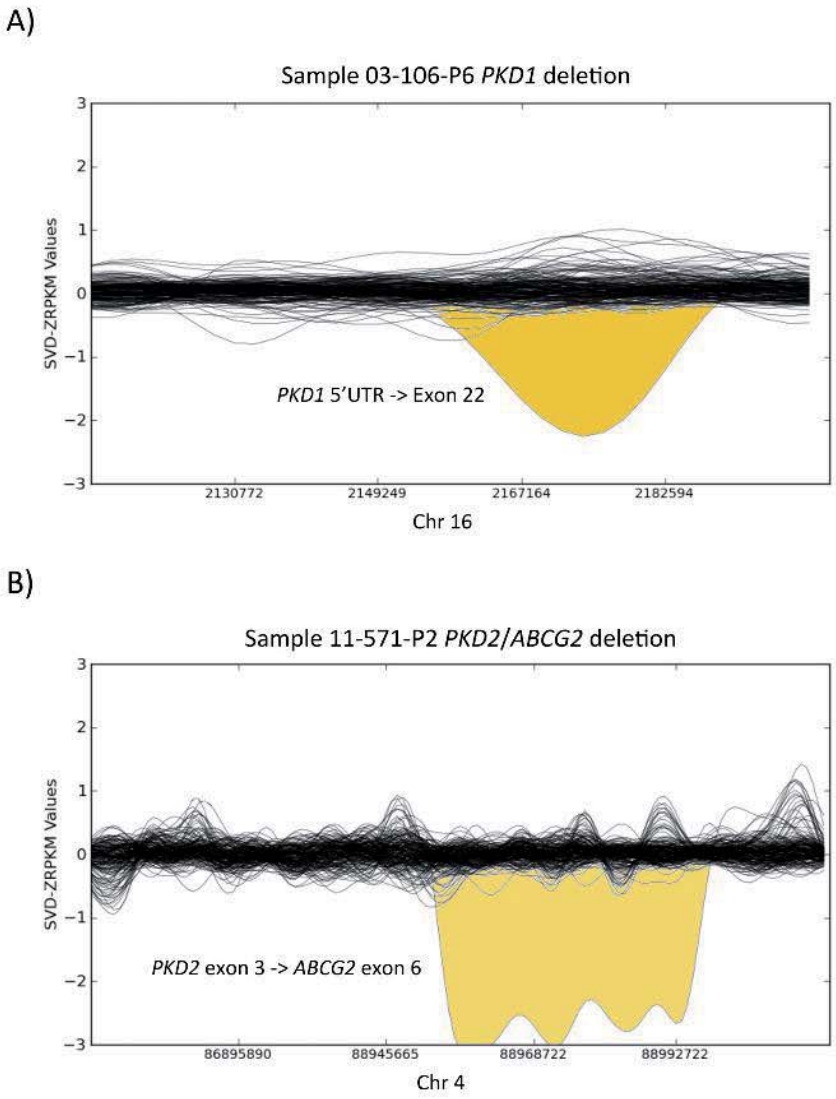

Figure 2. Detection of large deletions in the *PKD1* and *PKD2* genes by normalized depth of coverage analysis. Representation of the SVD-ZRPKM values calculated by Conifer for the 36 samples and 5 controls of the validation cohort. Yellow peaks indicate the two large deletions identified in this study. (A) Sample 03-106-P6 *PKD1*'s deletion (g.2154344-2186386del). (B) Sample 11-571-P2 *PKD2/ABCG2*'s deletion (g.88952828-89050618del).

method for the molecular diagnostics of ADPKD applying targeted NGS. First, we validated the assay in a cohort of 36 previously characterized ADPKD patients in which we detected 35 out of 36 known mutations. Second, we analyzed a discovery cohort of uncharacterized ADPKD patients and we reached a diagnostic rate of 83% (10 out of 12 patients), allowing test reporting 5 days after receiving the DNA samples. Although the size of our cohort is modest, these results are very encouraging since these numbers represent a diagnostic rate comparable to data obtained by Sanger sequencing (Audrezet et al. 2012; Cornec-Le Gall et al. 2013) and NGS (Rossetti et al. 2012).

Recently, targeted sequencing by NGS has been used in the identification of mutations in ADPKD. Rossetti et al. (2012) did not apply a capture protocol for *PKD1* and *PKD2* enrichment since they speculated that the duplicated genomic regions of *PKD1* would lead to concurrent capture of the six *PKD1* pseudogenes making very difficult the identification of the ADPKD causal variants. Instead, these authors used a strategy of pooling equimolar LR-PCR amplicons and multiplexing barcoded libraries. Their approach showed a high sensitivity, specificity and accuracy, but it is a very laborious task more amenable to characterize large ADPKD populations than for routine genetic diagnosis. Moreover, their approach did not allow

Table 3. ADPKD mutations in *PKD1* and *PKD2* identified in the 12 samples of the discovery cohort.

Sample	Gene	Duplicated region	cDNA change	Protein change	PKDB	# Patients	Classification	Ref counts	Variants counts
06-056	*PKD1*	Yes	c.348_352delTTTAA	p.(Asn116fs)	Present	1	Definitely pathogenic	28	20
06-122	*PKD1*	Yes	c.7204C>T	p.(Arg2402*)	Present	2	Definitely pathogenic	24	12
07-032	*PKD1*	Yes	c.8421_8422insC	p.(Ile2808fs)	Absent	0	Definitely pathogenic	22	18
11-444	*PKD1*	Yes	c.8041C>T	p.(Arg2681Cys)	Absent	0	Highly likely pathogenic	38	20
12-444	*PKD2*	–	c.1532_1533insAT	p.(Asp511fs)	Absent	0	Definitely pathogenic	156	70
	PKD1	–	c.10921C>T	p.(Arg3642Cys)	Absent	0	Highly likely pathogenic	40	52
12-505	*PKD1*	Yes	c.50174_5015delAG	p.(Arg1672fs)	Present	28	Definitely pathogenic	118	88
13-199	*PKD1*	Yes	c.7039delC	p.(Arg2347fs)	Absent	0	Definitely pathogenic	34	32
12-628	*PKD1*	Yes	c.2180T>C	p.(Leu727Pro)	Absent	0	Highly likely pathogenic	20	8
08-258	*PKD1*	Yes	c.7925C>T	p.(Arg2639*)	Present	5	Definitely pathogenic	28	14
10-484	*PKD1*	–	c.12010C>T	p.(Gln4004*)	Present	4	Definitely pathogenic	26	38
13-102	–	–	–	–	–	–	–	–	–
07-335	–	–	–	–	–	–	–	–	–

patients in previous studies, NM_001009944.2 for *PKD1* and NM_000297.2 for *PKD2*.

detecting large genomic rearrangements. Here, we do not only demonstrate that genome enrichment by in-solution hybridization using an elaborated probe design is an accurate strategy for mutation identification in the duplicated regions and the rest of *PKD1* and *PKD2*, but also that this strategy is ready to substitute LR-PCR-based methods in the routine genetic diagnostics of ADPKD to detect all sorts of sequence variants, including SVs.

When we conceived this study, we assumed that it would be extremely difficult to specifically capture the genuine *PKD1*, that is, there would always be residual enrichment of the six pseudogenes. Therefore, instead of excluding this region from our assay we decided to include unspecific probes to the duplicated region of *PKD1* in our capture library. In this regard, the mutation calls are on average lower than the reference calls, most likely due to the pseudogenes background (Tables 2, 3). From our point of view, the critical point of the assay was not the presence of sequencing reads coming from both the genuine *PKD1* and its pseudogenes. Instead, the major challenge was to map the reads coming from duplicated regions unambiguously to the genuine *PKD1* or to the six pseudogenes.

In order to minimize the impact of sequence reads coming from the pseudogenes we allowed mapping to the whole genome, instead of restricting the mapping to the targeted region. Moreover, the length of the millions of overlapping sequencing reads produced in this study (2×100 bp and 2×250 bp in the validation and discovery cohorts, respectively) in combination with the 300 bp insert sizes in the DNA libraries provided enough sequence specificity for accurate mapping and pseudogene discrimination, allowing us to unambiguously map a large proportion of the sequencing reads to *PKD1* (Table 1).

Furthermore, we also assume that the alignment algorithm is not 100% reliable and some reads coming from the pseudogenes could have been erroneously aligned to *PKD1*. In the worst scenario, the accumulation of these misaligned reads could lead to spurious variant calls but, as we have observed, none of these potential false positive variant calls passed the stringent filters of our variant prioritization pipeline in any of the patients of the validation and discovery cohorts neither in the five control samples.

The low sequencing coverage obtained for exons 1 and 42 of *PKD1* and exon 1 of *PKD2*, likely due to a high GC content, is the main limitation of this study as all variants that were missed were located in these poorly covered regions highlighting the importance of achieving sufficient depth of coverage for the optimal performance of the assay. However, we think that this can be fixed by rebalancing and adding new and replicate probes hybridizing with these poorly covered regions in the capture design. In the discovery cohort, the low average depth of coverage yielded by the MiSeq (Illumina, Inc.) in some samples was the cause of the lower mutation detection rate of the assay. However, we plan in the future to produce an optimized capture design including only *PKD1/PKD2* and a few cystic genes that would help in the differential diagnosis, such as *HNF1B* (17p12; OMIM ID: 189907) and *PKHD1* (6p12.3-p12.2; OMIM ID: 606702). This would significantly reduce the total captured DNA per sample, allowing multiplexing more samples per MiSeq (Illumina, Inc.) run, and to achieve higher depths of coverage (comparable to those obtained for the validation cohort) that will allow more confident variant calling.

We estimate that with our NGS-based assay a 60% of cost savings per sample could be achieved, and the whole diagnostics process could be a minimum of five times faster than with the conventional techniques currently used

for the genetic diagnostics of ADPKD. In addition, our strategy offers a complete definition of the captured genes, without the need for stepwise testing anymore and having to choose which gene to sequence first, and is capable to detect large genomic rearrangements and deep intronic variants. In the discovery cohort, the complete process of library preparation, genomic enrichment, NGS using a MiSeq (Illumina, Inc.), and bioinformatics analysis was completed in 5 days after reception of the DNA samples.

In conclusion, we illustrate here the first successful study using in-solution hybridization enrichment coupled to NGS to detect ADPKD pathogenic mutations, both in the duplicated regions of *PKD1* and the rest of *PKD1* and *PKD2* genes. Our approach is cost- and time-effective, and meets the sensitivity and specificity criteria required for genetic diagnostics, providing NGS experimental and bioinformatics approaches ready to substitute classic molecular tools in routine genetic diagnostics of ADPKD.

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Conflict of Interest

The authors declare that they have no competing interests and the results presented in this paper have not been published previously in whole or part, except in abstract format.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Target regions for capture of the *PKD1* and *PKD2* genes.

Table S2. Average coverage statistics of *PKD1* and *PKD2* exons in the validation cohort.

Table S3. By sample sequencing of quality control and coverage statistics in the validation cohort.

Table S4. By sample sequencing quality control and coverage statistics in the discovery cohort in sequencing of *PKD1* and *PKD2*.

Table S5. NGS of *PKD1* and *PKD2* versus genotyping calls in the five control samples.