Contribution of High-throughput sequencing to the Diagnosis of tubulopathies and Familal Hypercalcemia Hypocalciuria in Adults

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Abstract:

Hereditary tubulopathies are rare diseases with unknown prevalence in adults. Often diagnosed in childhood, hereditary tubulopathies can nevertheless be evoked in adults. Precise diagnosis can be difficult or delayed due to insidious development of symptoms, comorbidities and polypharmacy. The aim of this study was to evaluate the diagnostic value of a specific panel of known-genes implicated in tubulopathies in adult patients and compare with our data obtained in children. We analysed 1033 non-related adult patients with a clinical diagnosis of tubulopathy (n=744) or familial hypercalcemia with hypocalciuria (n=289) recruited by three European reference centres. Three-quarters of our tubulopathies cohort included individuals with clinical suspicion of Gitelman syndrome, renal hypophosphatemia and renal tubular acidosis. We detected pathogenic variants in 26 different genes confirming a genetic diagnosis of tubulopathy in 29% of cases. In In 16 cases (2.1%) the genetic testing changed the clinical diagnosis. The diagnosis of familial hypercalcemia with hypocalciuria was confirmed in 12% of cases. This work demonstrates the genetic origin of tubulopathies in one out of three adult patients, which is half of the rate observed in children. Establishing a precise diagnosis is crucial for patients, in order to guide care, to survey and prevent chronic complications as well as for genetic counselling. At the same time, this work enhances our understanding of complex phenotypes and enriches the database of causal variants described.

Keywords: Tubulopathy, Next Generation Sequencing, Adults, genetic testing.

Introduction

Inherited renal tubulopathies are rare diseases often diagnosed in children, particularly those with autosomal recessive transmission. Although some tubulopathies are diagnosed in adulthood, including recessive diseases with potentially mild presentation (e.g. Gitelman syndrome), slowly progressive dominant diseases, (e.g. Autosomal Dominant Tubulointerstial Kidney Disease), and diseases with variable severity (e.g. Dent disease), the prevalence of tubulopathies in adults remains mostly unknown.[1,2,3] In addition, the clinical presentation may be atypical or insidious, complicating and delaying the diagnosis of tubular dysfunction.[4]

Advances in understanding renal tubular solute transport systems has been achieved through the elucidation of monogenic tubular disorders, and follow-up studies using cellular and animal models.[5] In turn, these advances allowed to improve molecular diagnosis and genetic counselling, as well as to devise therapeutic or preventive measures.[6] The introduction of high-throughput gene panel sequencing makes the analysis of several genes at the same time possible for genetically heterogeneous diseases and allows accurate diagnosis in patients with atypical presentations.[7]

We recently published the experience of three European centres using a next generation sequencing (NGS) panel of 37 known genes to assess children with suspected tubulopathies and confirmed genetic disease in two-thirds of those tested.[8] In this article, we describe our results in a large adult population using the same panel as well as an improved second version containing nine additional genes.

Results

Patients

The clinical diagnoses of the adult cohort of 1033 patients comprise 20 different tubulopathies and Familial Hypercalcemia Hypocalciuria (FHH) (Table 1) . Four diagnoses correspond to 75% of the cohort: FHH, N=289, Gitelman syndrome (GS, N=272), renal hypophosphatemia (RH, N=124), which includes nephrolithiasis/osteoporosis hypophosphatemic (NPHOP) and Hereditary Hypophosphatemic Rickets with Hypercalciuria (HHRH), and distal Renal Tubular Acidosis (dRTA, N=86). Details of additional suspected diagnoses and their proportion in the cohort are summarized in Table 1.

Overall diagnostic performance: initially suspected and diagnostic revision:

In this cohort, pathogenic or likely pathogenic variants were detected in 26% of cases (269/1033) allowing a genetic confirmation. In 24.5% (253/1033) these variants were directly related to the diagnosis initially suspected. In contrast, in 1.5% (16/1033) the genetic findings corresponded to a different diagnosis, symptoms of which may phenocopy the suspected initial pathology. If we consider only tubulopathies, the revision rate is 2.1% (16/744). The global performance is summarized in Supplementary Figure 1.

Genetic variants

A total of 275 variants were detected in 26 out of the 46 genes included in version 2 of the panel; a third of these variants were not previously described (Table 2). 86% of the variants (236/275) were classified as class 4 or 5 and 25% (59/236) were not previously described. Table 3 summarizes novel variants classified as class 4 or 5 along with the criteria used for

their classification. The variants already reported as well as class 3 variants are summarized by disease and by gene in Supplementary Table 1.

The analysis of 90 relatives from 56 different families allowed confirmation of: (i) the genetic diagnosis in 29 affected relatives; (ii) compound heterozygosity in patients with recessive diseases after the analysis of parents or children in 22 families, (iii) confirmation of heterozygous carrier status in two cases of X-linked disease; and exclusion of familial disease in 15 relatives of patients with autosomal dominant disorders.

Performance by disease:

The detection rates of mutations by disease entity are shown in Figure 1A.

Familial Hypercalcemia Hypocalciuria

FHH constitutes the largest part of our cohort (28%, 289/1033 patients). Patients with this diagnosis were mainly included by the Paris centre. Among patients with this clinical diagnosis, 12.5% (36/289) were genetically confirmed. Mutations were identified in the *CASR* gene in 81% (29/36 FHH1), in the *AP2S1* gene in 14% (5/36 FHH3) and in the *GNA11* gene in 6% (2/36 FHH2). The available clinical and biochemical characteristics of these patients are summarized in supplementary Table 2.

Tubulopathies

GS represents the ~~second~~ most important clinical diagnosis of the tubulopathies in this cohort (26%, 272/1033). Among these, 43% (117/272) were genetically confirmed, i.e. detection of two heterozygous class 4 or 5 variants in the *SLC12A3* gene.

RH corresponds to 12% of the cohort (124/1033) with a mutation detection rate of 18% (22/124). Thirteen patients had variants in the *SCL34A3* gene: 8 had one heterozygous variant, and 5 had two heterozygous variants (unfortunately DNA from relatives was unavailable for segregation studies). Seven patients had heterozygous pathogenic variants in the *SLC9A3R1* gene. Finally, two patients had heterozygous pathogenic variants in the *SLC34A1* gene. Interestingly one of patients with clinical and genetic diagnosis of RH with pathogenic heterozygous variant *SCL34A3* has also a class 5 variant in the *SLC9A3R1* gene, raising the possibility of digenism.

The third most common tubulopathy in the cohort is dRTA, representing 8% of the patients (86/1033). Out of the 86 patients with this diagnosis, 18 were genetically confirmed (21%). *SLC4A1* was implicated in 15 patients (83%), *ATP6V1B1* in 2 patients, and *ATP6V0A4* in 1 patient.

Autosomal Dominant Tubulointerstitial Kidney Disease related to *UMOD* (ADTKD-*UMOD*), represents 4.5% of the cohort (47/1033), and 17% of these patients (8/47) had genetic confirmation. Dent disease represents 3.6% of the cohort (37/1033), with a rate of genetic confirmation of 49% (18/37). Fifteen patients carried *CLCN5* variants (Dent 1), and 3 patients carried *OCRL* variants (Dent 2). BS and Autosomal Dominant Hypocalcaemia (ADH) each represent 3% of the cohort (31 and 34/1033) with a rate of genetic confirmation of 20% (6/31) and 9% (3/34) respectively.

The following diagnoses each represent less than 3% of the cohort and have a rate of genetic confirmation between 18 and 50% (Table 1): Pseudohypoaldosteronism type 2 (PHA2), hypomagnesemia (HOMG), Infantile Hypercalcemia (IH) and renal glycosuria (RG). Finally,

one patient with clinical diagnosis of mixed RTA had a genetic confirmation (homozygous class 4 variant in the *CA2* gene).

Other clinical diagnoses, for which no genetic confirmation was made included: patients with type 1 pseudohypoaldosteronsim (PHA1), nephrocalcinosis (NC), hypertension, nephrogenic diabetes insipidus, nephrogenic syndrome of inappropriate antidiuresis (NSIAD), Fanconi syndrome, hypouricemia and hyperuricemia (Table 1).

Patients with variants of unknown significance (VUS)

Thirty variations detected in 16 different genes in 40 patients were classed as class 3 or VUS. They are described by disease and by gene in Supplementary Table 1. In ten patients with clinical BS or GS, the class 3 variant was associated with a class 4 or 5 variant in the same gene.

Genetic revision of the clinical diagnosis

In 16 cases, the analysis of other genes present in the panel allowed a revision of the diagnosis. Table 4 summarises these cases.

First, half of these cases correspond to the known overlap of phenotypes of GS and classic BS that turned out to be genetically either BS type 3 or GS.

Second, one patient with clinical diagnosis of ADTKD had a genetic diagnosis of BS type 1 (patient B26). This patient had a diagnosis of chronic kidney disease at the age of 32 years, in absence of other manifestations except polyuria; she had hyperuricemia and normal level of potassium. Her oldest sister had gouty arthritis before the age of 30 years. The patient and her younger sister had nephrocalcinosis. DNA samples were not available to test family segregation.

Third, 3 cases with clinical diagnosis of incomplete dRTA were genetically diagnosed as type 2 BS (patient L84), GS (L150) and RH (P6). Patient L84 had hypokalaemia and nephrocalcinosis diagnosed age 35 yr. She was referred to nephrology 3 years later, on potassium supplements and amiloride. Her parents were first cousins and she had a strong family history of kidney stones. Plasma bicarbonate at presentation was 26 mmol/L, and the initial clinical diagnosis was of incomplete dRTA. L150 is a patient from Turkish Cyprus, who had severe acidosis (bicarbonate 12 mmol/L), hypokalaemia (potassium 2.7-3 mmol/L), normal plasma magnesium and chloride and a urinary pH of 8. He was on potassium supplements and spironolactone at the time of genotyping. Patient P6 had a history of kidney stones and hypocitraturia; an acid load test showed an inappropriate urinary pH (5.8) and an abnormal ammonia excretion establishing the diagnosis of incomplete dRTA, nevertheless a borderline phosphate concentration was also observed; in this patient two heterozygous variant in *SLC34A3* were detected.

Fourth, the patient with renal hypomagnesemia who had a final diagnosis of GS (P113) presented with severe hypomagnesemia (0.25 mmol/L) and borderline hypokalemia (3.4 to 3.9 mmol/l); the hypokalemia was initially considered secondary to hypomagnesemia.

Fifth, in one patient with clinical diagnosis of Dent disease (P179), the genetic diagnosis was an autosomal dominant dRTA. This patient with a history of stone disease was given a diagnosis of Medullary Sponge Kidney disease at 20 years old; an evaluation three years later showed proximal tubulopathy and CKD: renal hypophosphatemia, hyperchloremic metabolic acidosis, hypocitraturia, proteinuria (1.41g/day with moderate albuminuria 143 mg/day) and Inulin clearance of 58 ml/min/1.73 m^2. No variants were detected in genes responsible for Dent disease or renal Fanconi syndrome. Taking into account the presence of stone disease

and hypocitraturia, we looked for variants in genes responsible of dRTA and detected a known class 5 variant in the *SLC4A1* gene.

Finally, two cases with clinical diagnosis of IH and nephrolithiasis had heterozygous variants in the *SLC34A3* gene (B61 and P582).

DISCUSSION

Hereditary tubulopathies are less frequently diagnosed in adults. However, the establishment of a precise diagnosis is important to guide care, survey and potentially prevent chronic complications as well as for genetic counselling. In this paper we describe the analysis of a large multicentre cohort of adult patients with a panel of 46 genes involved in tubulopathies or in FHH, which yielded an overall genetic confirmation in 26% of cases after the detection of pathogenic variants in 26 different genes.

Twenty-eight percent of cases of this cohort have a clinical diagnosis of FHH, which is not primarily a tubulopathy. The genes responsible for this disease have been included due to the role of the corresponding proteins in tubular calcium reabsorption. In addition, patients with this diagnosis were mainly included by Paris centre. With the exclusion of this disease, the rate of genetic confirmation increases to 29% (216/744), which is approximately half of the rate we observed previously in children (64%).[8] This percentage remains unchanged when genes presented only in the 2nd panel version are excluded (see Supplementary Table 3 for panel composition).

A recent study analysed a panel of 30 genes that cause nephrolithiasis/nephrocalcinosis (NC/NL) in a cohort of children and adults showing that the detection rate of monogenic causes in adults was half that seen in children (11.4% vs 20.8%).[9]

Several features may explain the lower rate observed in adults. First, autosomal recessive tubulopathies are mainly diagnosed in children. Accordingly, Halbritter et al., in their study of monogenic causes of NC/NL found that recessive causes were diagnosed more frequently in childhood, whereas dominant disease more commonly in adulthood.[9] Nevertheless, in our cohort autosomal recessive diseases were confirmed in 153/269 patients (57%), primarily in patients with GS (n=120) and BS (n=16).

Second, the presence of co-morbidities and polypharmacy in cohorts of adult patients can make interpretation of the phenotype more difficult (especially when the phenotype involves chronic kidney disease), or complicate the interpretation of urinary chemistry. Also acquired tubular dysfunction (e.g. autoimmune dRTA, renal Fanconi syndrome caused by drug or environmental toxicity) is much more common in adults and genetic testing may be an important part of investigation for these patients.[10, 11, 12]

Third, a significant number of tests are performed as exclusion diagnosis. This is especially true for patients with clinical diagnosis of FHH, for which we have a low diagnosis rate (12.5%). FHH could have a very similar clinical presentation to primary hyperparathyroidism (PHPT) and, despite proposed scores and algorithms, it remains difficult to have a precise clinical diagnosis.[13] It is therefore recommended in these cases to exclude a diagnosis of FHH before committing to parathyroidectomy, which is inappropriate in FHH.[14] We were able to analyse the data of 86 patients for whom a genetic diagnosis of FHH was excluded and follow-up information was available. Thirty-five patients (41%) were parathyroidectomised and 31% of them (11/35) normalised their serum calcium level post-operatively while the remaining 24 patients had persistent hypercalcemia. This percentage is higher than the usual rate of unsuccessful parathyroidectomy in primary hyperparathyroidism (5-10%). The

diagnosis of FHH is not completely excluded in this population because there are probably other genes responsible for this disease.[15] This highlights the importance of pursuing further research genetic investigations to identify other genes responsible for these rare phenotypes. Concerning the clinical presentation of different types of FHH, although there are few cases of FHH2 and FHH3 in this cohort; analysis of clinical data (Supplementary Table 2) shows similar results to results described in the literature, i. e. higher hypercalcemia in patients with FHH3.[16, 17]

Gitelman syndrome was the most common tubulopathy in adults with a high molecular confirmation rate (46%, Figure 1). The main symptoms of GS are rather nonspecific (cramps, poorly defined discomfort, fatigue) and they evolve slowly, resulting in a late or incidental presentation. While the biochemical phenotype is typically well characterized, explaining the high level of genetic diagnosis, patient L150, presenting with acidosis, demonstrates that it can be surprisingly variable. Notably, in 55% of patients with available clinical information (42/76), the genetic test was performed to exclude the diagnosis (e.g. in patients with eating disorders or surreptitious diuretic abuse and questionable biological data) and as expected no variants were detected.[18]

In contrast with GS, Bartter syndrome the most common tubulopathy in children is rarely diagnosed in adulthood. BS was confirmed in 16 patients. As expected most of them (n=12) had BS type 3. Three patients had genetic diagnosis of BS type 1 (P103, B26, B46) and one patient BS type 2 (L84). B26 and L84 patients correspond to revised diagnosis. Patient P103 had a diagnosis of hypokalaemia made when she was 30 years old on routine examination during her first pregnancy; later evaluation revealed alkalosis, high renin level and hypercalciuria. She harboured two missense class 4 heterozygous variants in *SLC12A1*

located in *trans* (her son is heterozygous for one of two variants). The presentation in the woman B46 was quite similar with reduced serum potassium concentration at age of 35 years with mild hypomagnesemia and hypercalciuria. The patient was heterozygous for a class 4 missense change and a complete deletion of exon 16. This rearrangement was expected to lead to frameshifted transcript. Whereas BS type 1 is in general associated with polyhydramnios and severe neonatal manifestations, there is a description of late-onset manifestations related with partial loss of function of the mutant.[19] A similar situation could explain the late-onset in these patients, which needs confirmation by *in vitro* studies.

Among patients with clinical BS and GS, 10 cases harboured two variants but one of them was considered as class 3; for these variants there are no criteria for benignity and the confirmation that they are located in *trans* after familial segregation studies will allow to reclass them as class 4 increasing the confirmation rate.

NL/NC is a common clinical manifestation in adult patients. The main monogenic causes of NL/NC are: Dent disease, RH, complete and incomplete dRTA, and IH representing 17% of cases with genetic confirmation in our cohort. For patients with clinical diagnosis of Dent disease we had a higher genetic confirmation rate in adults than in children (49% versus 40%). This may be explained by the incomplete renal Fanconi syndrome that they have as infants, easy to miss clinically, and the fact that nephrocalcinosis and renal failure may only become evident during adult life. Causative genes were *CLCN5* in 83% of cases, and *OCRL* in 17%, similar to that described in the literature.[20] The contribution of molecular diagnosis for these patients is crucial, given the progression to end-stage kidney disease (typically in the 4^{th} or 5^{th} decade of life) allowing genetic counselling and detection of heterozygous female carriers. For RH and dRTA we observed a relatively lower detection rate that interestingly is

similar to the rate described by Halbritter et al in adults.[9] This lower detection rate may be explained by the multifactorial origin of NL/NC but we cannot exclude variants not detected by our method (i.e. deep intronic variants), known genes not present in our panel (i.e. *SLC7A9*, *ADCY10*) or a supplementary genetic heterogeneity.[9, 21] In 5 patients with nephrolithiasis, we detected 2 pathogenic heterozygous variants of the *SLC34A3* gene; unfortunately DNA from relatives was unavailable to confirm that they are bi-allelic; 2 out 5 of these patients presented with osteopenia and two other with nephrocalcinosis. Finally, type 1 Infantile Hypercalcemia was confirmed in 3 patients. Previous reports and this data confirm that IH can also be an adult disease. Heterozygous patients can have hypercalciuria and nephrolithiasis as well as high calcitriol levels.[22, 23] In one of our index cases (P580) only one heterozygous class 5 variant was detected. Although we cannot exclude the presence of a second variant in a non-analysed region of the gene; this observation rises also the possibility of clinical manifestation in heterozygous patients.

In PHA2, we found a high rate of confirmed diagnoses (39%), mainly explained by pathogenic variants in *KLHL3.* The biochemical phenotype of PHA2 (hypertension, hyperkalaemia and metabolic acidosis) is quite specific, which may explain this high rate. KLHL3 molecular abnormalities explain a high proportion of patients with this disease in the cases described in the literature.[24]

For the patients with HOMG suspected of genetic origin, 2 patients had hypomagnesemia related to TRPM6 mutations (HOMG1). This syndrome is usually diagnosed during the first year of life, with convulsions associated with severe hypocalcaemia.[25] Patient P555 from a consanguineous family had a diagnosis of hypomagnesemia secondary to intestinal

malabsorption at 32 years old; she had secondary hypoparathyroidism and no neurological manifestations, again demonstrating the spectrum of clinical severity.

The rate of discrepancy between clinical and genetic diagnosis is relatively low (2.1 %), which underlines the diagnostic accuracy of the referring clinicians. A total of 16 patients had their initial clinical diagnosis revised by the panel analysis. As in children, the revision was rarely substantial and mostly concerns salt losing tubulopathies and/or diseases associated with nephrocalcinosis/nephrolithiasis. Several tubulopathies present with either hypokalaemia and/or hypercalciuria and thus can constitute phenocopies of these findings (Figure 2).

The most common revision was from GS to BS type3 or *vice versa* in 8 cases, which was expected taking into account the well-known phenotypic overlap between these 2 syndromes. Three cases with clinical diagnosis of incomplete dRTA were found to type 2 BS, GS and RH (L84, L150, P6) respectively. Two cases with clinical diagnosis of IH and nephrolithiasis had heterozygous variants in the *SLC34A3* gene (B61 and P582). This interesting observation shows that patients with absorptive hypercalciuria due to increased calcitriol as in IH or renal hypophosphatemia can have overlapping phenotypes and clinical and biological manifestations in heterozygous.[22, 23, 26] For patient B26 with genetic diagnosis of BS type 1, the clinical diagnosis of ADTKD could have been excluded due to the presence of nephrocalcinois.[2] Finally, in one patient with clinical diagnosis of Dent disease (P179) the genetic diagnosis was an autosomal dominant dRTA.

In conclusion, this work demonstrates that a genetic cause can be demonstrated in one out of three adult patients presenting with a clinical suspicion of tubulopathy. This rate of detection is half of that observed in children. The establishment of a genetic diagnosis is crucial for the patient, in order to guide care, to survey and prevent chronic complications as

well as for genetic counselling. These results enhance our understanding of complexes phenotypes and enrich the database of causal variants associated with clinically-defined tubulopathies.

Patients and Methods

Patients:

A total of 1033 adult index cases with a clinical diagnosis of tubular dysfunction made after the age of 18 years and for which the implicated gene(s) are present in the panel were included and analysed from 2014 to 2016. The clinical diagnosis was performed by physicians belonging to the network of three expert centres. Of these, 139 samples were analysed in Brussels, 171 in London and 723 in Paris. The last centre has a large recruitment of patients with hypercalcemia of parathyroid origin, explaining the inclusion of a large number of patients with suspected Familial Hypercalcemia Hypocalciuria, which is not strictly speaking a tubulopathy. In addition, 90 relatives belonging to 56 families in whom a pathogenic variant was detected in the proband were analysed; these included 29 affected individuals. Informed consent for genetic testing was obtained by the respective treating physician after approval by the respective institutional review boards, in accordance with the Declaration of Helsinki.

Gene amplification, sequencing and bioinformatic analysis:

This was performed as described previously.[8] Briefly, genomic DNA was isolated from white blood cells using standard procedures, followed by massive parallel sequencing using the versions 1 and 2 of a tubulopathies panel designed by the work package tubulopathies of the European consortium EURenOmics. The 1st version was previously described in our

paediatric cohort.[7] The 2nd version includes 9 additional genes; the Supplementary Table 3 compares the composition of these two versions

The depth for each region of interest was at least 30X, except for exon 1 of *OCRL* and *WNK1*, analysis of which was completed by Sanger sequencing in those with suspected disease related to these 2 genes and with no other causative mutation identified. Regions with coverage lower than 30X were sequenced by Sanger. Library preparation and bioinformatics analysis was performed according to the technics and pipelines routinely used by each laboratory (Supplemental methods). The variants were classified according to the guidelines published by the American College of Medical Genetics ACMG 2015.[27] Variants of interest were verified by Sanger sequencing.

The three centres are accredited laboratories proposing genetic diagnostic tests referenced in Orphanet (https://www.orpha.net/consor/cgi-bin/index.php?lng=EN or https://ukgtn.nhs.uk/find-a-test/search-by-laboratory/laboratory/london-north-east-rgc-gosh-43/)

DISCLOSURE

AR and JDF are employees of Multiplicom, provider of the kits used for amplification of the 46 genes. All the other authors declared no competing interests.

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Table 1. Clinical and genetic diagnoses in the cohort of 1033 adult subjects.

Clinical diagnoses	Patient number (%)	Genetic diagnoses (%)	Diagnosis revision [n]	% Global diagnostic rate (confirmed+revised)
FHH	289 (28)	36 (12.5)	0	12.5
GS	272 (26)	117 (43)	8	46
RH	121 (12)	22 (18.2)	0	18.2
dRTA [§]	86 (8)	18 (20.9)	3	24.4
ADTKD	47 (4.5)	8 (17)	1	19.1
Dent	37 (3.6)	18 (48.6)	1	51.4
BS	30 (3)	6 (20)	1	23.3
ADH	34 (3)	3 (8.8)	0	8.8
PHA2	28 (2.7)	11 (39.3)	0	39.3
HOMG	24 (2.3)	2 (8)	1	12.5
IH	15 (1.5)	3 (20)	1	26.7
RG	15 (1.5)	7 (47)	0	47
NC or FNL	10 (1)	1 (10)	0	10
NDI	3 (0.3)	0 (0)	0	0
PHA1	1 (0.1)	0 (0)	0	0
Mixed RTA	1 (0.1)	1 (100)	0	100
Hypertension[$]	2 (0.2)	0	0	0
NSIAD	4 (0.4)	0	0	0
Fanconi	2 (0.2)	0	0	0
Hyperuricemia	2 (0.2)	0	0	0
Hypouricemia	2 (0.2)	0	0	0
Total	**1033**	**253 (24.5)**	**16**	**26**

[§]Complete and incomplete. [$] With hypo or normokalemia

FHH, familial hypercalcemic hypocalciuria; GS, Gitelman syndrome; RH, renal hypophosphatemia (including hypophosphatemic rickets); dRTA, distal renal tubular acidosis; ADTKD, autosomal dominant tubulointerstitial kidney disease; Dent, Dent disease; BS, Bartter syndrome; ADH, autosomal dominant hypocalcemia; PHA2, pseudohypoaldosteronism type 2; HOMG, hypomagnesemia; IH, Infantile hypercalcemia; RG, renal glycosuria; NC or FNL, nephrocalcinosis or familial nephrolithiasis; NDI, nephrogenic diabetes insipidus; PHA1, pseudohypoaldosteronism type 1; mixed RTA, mixed renal tubular acidosis; NSIAD, nephrogenic syndrome of inappropriate anti-diuresis; Fanconi, renal Fanconi syndrome.

Table 2. Molecular diagnosis

		Patients			Variants[£]	
Disease (#MIM)	**Gene**	**Diagnostic confirmed***	**Not confirmed****	**Total**	**Previously reported**	**Novel**
FHH1 (145980)	*CASR*	29	2	31	14	16
FHH3 (600740)	*AP2S1*	5	1	6	2	1
FHH2 (145981)	*GNA11*	2	1	3	0	3
GS (263800)	*SLC12A3*	120	23	143	92	18
RH (612286	*SLC34A1*	2	2	4	2	2
241530	*SLC34A3*[@]	16	3	19	13	10
612287)	*SLC9A3R1*	7	1	8	2	1
dRTA (267300)	*ATP6V1B1*	2	0	2	2	0
(602722)	*ATP6V0A4*	1	2	3	3	1
(179800)	*SLC4A1*	16	0	16	6	2
ADTKD (162000)	*UMOD*	8	1	9	3	6
Dent 1 (300009)	*CLCN5*	15	1	16	13	1
Dent 2 (300555)	*OCRL*	3	0	3	3	0
BS1 (601678)	*SLC12A1*	3	2	5	1	7
BS2 (241200)	*KCNJ1*	1	1	2	1	0
BS3 (607364)	*CLCNKB*	12	6	18	9	7
ADH (601198)	*CASR*	3	0	3	2	1
PHA2 (614495)	*KLHL3*	9	0	9	1	7
(614492)	*WNK1*	1	1	2	0	2
(614491)	*WNK4*	1	0	1	1	0
FHHNC (248250)	*CLDN16*	1	0	1	1	0
HOMG1 (602014)	*TRPM6*	1	0	1	0	1
IH 1 (143880)	*CYP24A1*	3	1	4	2	1
IH 2 (616963)	*SLC34A1*	0	1	1	0	1
RG (233100)	*SLC5A2*	7	2	9	6	5
pRTA (604278)	*SLC4A4*	0	1	1	0	1
mixed RTA (259730)	*CA2*	1	0	1	1	0
Hypouricemia (220150)	*SLC22A12*	0	1	1	0	1
Total	26 genes	269	53	322	180	95

*Patients with class 4 and 5 variants. **Patients with class 3 variants or only one heterozygous variant in a recessive disease. One patient of this group has also a pathogenic variant in the *SLC9A3R1* gene. [£]Variants of class 3 are included

FHH1, FHH2, FHH3, familial hypercalcemic hypocalciuria type 1, 2 and 3; GS, Gitelman syndrome; RH, renal hypophosphatemia (including hypophosphatemic rickets); dRTA distal renal tubular acidosis; ADTKD, autosomal dominant tubulointerstitial kidney disease; Dent 1 and 2, Dent disease type 1 and type 2; BS1, BS2

and BS3, Bartter syndrome types 1, 2, and 3; ADH, autosomal dominant hypocalcemia; PHA2, type 2 pseudohypoaldosteronism; FHHNC, familial hypomagnesemia with hypercalciuria and nephrocalcinosis; HOMG1 hypomagnesemia type 1; IH1 and IH2, infantile hypercalcemia types 1 and 2; RG, renal glycosuria; pRTA, proximal renal tubular acidosis; mixed RTA, mixed renal tubular acidosis; MIM, Mendelian Inheritance in Man, http://omim.org.

Table 3. Novel class 4 and 5 variants detected in this study and their classification according to ACMG

Gene	Nomenclature cDNA	Nomenclature protein	ACMG Class	Criteria
CASR	c.226T>C	p.Phe76Leu	4	PM1, PM2, PP2, PP3
	c.293T>C	p.(Phe98Ser)	4	PM1, PM2, PP2, PP3
	c.503C>A	p.(Ala168Asp)	4	PM1, PM2, PP2, PP3
	c.811T>C	p.(Ser271Pro)	4	PM1, PM2, PM5, PP2, PP3
	c.1104_1105del	p.(Leu368Phefs*17)	5	PVS1, PM1, PM2
	c.1345T>C	p.(Cys449Arg)	4	PM1, PM2, PP2, PP3
	c.1823G>A	p.(Trp608*)	5	PVS1, PM1, PM2
	c.2011_2020del	p.(Glu671ThrfsTer24)	5	PVS1, PM1, PM2
	c.2048C>T	p.(Ala683Val)	4	PM1, PM2, PP2, PP3
	c.2087T>C	p.(Leu696Pro)	4	PM1, PM2, PP2, PP3
	c.2159_2160dup	p.(Leu721Glyfs*21)	5	PVS1, PM1, PM2
	c.2188C>T	p.(Leu730Phe)	4	PM1, PM2, PP1, PP2, PP3
	c.2336A>G	p.(Tyr779Cys)	4	PM1, PM2, PP2, PP3
	c.2572T>C	p.(Tyr858His)	4	PM1, PM2, PP2, PP3
	c.2730del	p.(Ser911Profs*28).	5	PVS1, PM1, PM2
CLCNKB	c.577-8T>G	p.(?)	4	PM2, PM3, PP1, PP3
	c.(866+1_867-1)_(2016+1_2017-1)del	p.(?)	5	PVS1, PM1, PM2
	c.1297+G>A	p.(?)	4	PVS1, PM2, PP3, PP5
	c.577-8T>G	p.(?)	4	PM2,PM3,PP1,PP3
CYP24A1	c.612C>G	p.(Tyr204*)	5	PVS1, PM1, PM2, PP3, PP4
GNA11	c.49_57del	p.(Glu17_Lys19del)	4	PM1, PM2, PM4
	c.-12_53del	p.(?)	5	PVS1, PM1, PM2
KLHL3	c.233T>C	p.(Met78Thr)	4	PM2, PM5, PP2, PP3, PP4
	c.234G>A	p.(Met78Ile)	4	PM1, PM2, PM5, PP2, PP3
	c.444T>A	p.(His148Gln)	4	PM1, PM2, PP2, PP4
	c.922G>A	p.(Gly308Ser)	4	PM2, PP1, PP2, PP3, PP4
	c.1205T>C	p.(Phe402Ser)	5	PM1, PM2, PP1, PP2, PP3, PP4
	c.1216A>G	p.(Thr406Pro)	4	PM1,PM2,PP1,PP2
	c.1300G>A	p.(Val434Met)	4	PS4,PM1, PM2, PP2
SLC12A1	c.1493C>T	p.(Ala498Val)	4	PM1, PM2, PM3, PP5
	c.1875G>T	p.(Trp625Cys)	4	PS4, PM1, PM2, PP3
	c.1878G>A	p.(Trp626*)	5	PVS1, PM1, PM2
	c.2035A>G	p.(Asn679Asp)	4	PM1, PM2, PM3, PP1
	c.2873+1del	p.(?)	5	PVS1, PM1, PM2, PP3
SLC34A1	c.1222G>A	p.(Val408Met)	4	PM1, PM2, PM5
SLC34A3	c.496G>A	p.(Gly166Ser)	4	PS4,PM2, PP3
	c.926-2A>C	p.(?)	5	PVS1, PM1, PM2, PP3

	c.1361A>G	p.(Asn454Ser)	4	PS4, PM1, PM2, PP3
	c.1717_1732del	p.(Asn573Argfs*63)	5	PVS1, PM2
SLC12A3	c.658_663del	p.(Gly220_Leu221del)	4	PM1, PM2, PM4
	c.1670-8_1670-7delinsCA	p.(?)	4	PM2, PP1, PP3, PP4
	c.1687C>T	p.(Gln563*)	5	PVS1, PM2, PM3, PM4, PP1
	c.1861T>G	p.(Tyr621Asp)	4	PM1, PM2, PP1, PP3
	c.2186G>A	p.(Gly729Asp)	4	PM1, PM2, PM3, PM5, PP3, PP5
	c.2368+1del	p.(?)	5	PVS1, PM2, PP3
	c.2711T>A	p.(Ile904Asn)	4	PM1, PM2, PM5
SLC4A1	c.2703_2713del	p.(Asp902Argfs*14)	5	PVS1, PM1, PM2
	c.2716G>T	p.(Glu906*)	5	PVS1, PM1, PM2
SLC4A4	c.1107dup	p.(Ile370Tyrfs*2)	4	PVS1, PM2
SLC5A2	c.394C>T	p.(Arg132Cys)	4	PM2, PP1, PP3, PP4, PP5
	c.1450-1G>A	p.?	5	PVS1, PM2, PP3, PP4
	c.1639_1640dup	p.(Thr548AlafsTer50)	5	PVS1, PM2, PP3, PP4
TRPM6	c.278A>G	p.(Asp93Gly)	4	PM2,PM3, PP1, PP4
UMOD	c.179G>C	p.(Gly60Ala)	4	PM1, PM2, PP3, PP4
	c.478G>C	p.(Asp160His)	4	PM1, PM2 PP3 PP4
	c.692T>C	p.(Leu231Pro)	4	PM1, PM2, PP3, PP4
	c.851T>C	p.(Leu284Pro)	4	PM1, PM2, PP3, PP4
	c.274T>C	p.(Cys92Arg)	4	PM1, PM2, PM (segregation)
WNK1	c.1888G>A	p.(Glu630Lys)	4	PM1, PM2, PP1, PP3, PP4

ACMG criteria description: PVS: very strong evidence of pathogenicity. PVS1: null variant (nonsense, frameshift, canonical +/−1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where loss of function is a known mechanism of disease. PS: strong evidence of pathogenicity. PS1: same amino acid change as a previously established pathogenic variant regardless of nucleotide change. PS3: well-established in vitro or in vivo functional studies supportive of a damaging effect on the gene or gene product. PS4: the prevalence of the variant in affected individuals is significantly increased compared to the prevalence in controls. PM: moderate evidence of pathogenicity. PM1: Located in a mutational hot spot and/or critical and well-established functional domain without benign variation. PM2: absent from controls (or at extremely low frequency if recessive) in gnomAD database. PM3: detected *in trans* with a pathogenic variant (the phase was determined). PM4: protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants. PM5: Novel missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before. PP: supporting evidence of pathogenicity. PP1: co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease. PP2: Missense variant in a gene that has a low rate of benign missense variation and in which missense variants. PP3: Multiple lines of computational evidence support a deleterious effect on the gene or gene product. PP4: Patient's phenotype or family history is highly specific for a disease with a single genetic aetiology. PP5: Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation.

Table 4. Index Cases with genetic revision of the clinical diagnosis

Patient	Clinical Diagnosis	Genetic Diagnosis
L81	Gitelman	Bartter 3
L117	Gitelman	Bartter 3
L123	Gitelman	Bartter 3
P56	Gitelman	Bartter 3
P104	Gitelman	Bartter 3
P116	Gitelman	Bartter 3
P608	Gitelman	Bartter 3
L85	Bartter 3	Gitelman
B26	ADTKD	Bartter 1
L84	Incomplete dRTA	Bartter 2
L150	Incomplete dRTA	Gitelman
P6	Incomplete dRTA	Renal hypophosphatemia
P113	Hypomagnesemia	Gitelman
P179	Dent	dRTA
B61	Familial nephrolithiasis	Renal hypophosphatemia
P582	Infantile Hypercalcemia	Renal hypophosphatemia

ADTKD, autosomal dominant tubulointerstitial kidney disease; dRTA, distal renal tubular acidosis

Figure Legends

Figure 1: Pie charts (n;%) and bar graphs comparing detection rates, distribution of genetically confirmed pathologies and confirmation rate by disease in (A) adults and (B) children

Figure 2: Tubulopathies phenocoping two frequent clinical presentations in the adult cohort. Dent, Dent disease; Bartter, Bartter syndrome; FHHNC, familial hypomagnesemia with hypercalciuria and nephrocalcinosis; ADH, autosomal dominant hypocalcemia; Liddle, Liddle syndrome.