

The genetic and clinical spectrum of a large cohort of patients with distal renal tubular acidosis



Viviana Palazzo^{1,21}, Aldesia Provenzano^{1,21}, Francesca Becherucci², Giulia Sansavini², Benedetta Mazzinghi², Valerio Orlandini¹, Laura Giunti³, Rosa Maria Roperto², Marilena Pantaleo³, Rosangela Artuso³, Elena Andreucci³, Sara Bargiacchi³, Giovanna Traficante³, Stefano Stagi⁴, Luisa Murer⁵, Elisa Benetti⁵, Francesco Emma⁶, Mario Giordano⁷, Francesca Rivieri⁸, Giacomo Colussi⁹, Silvana Penco¹⁰, Emanuela Manfredini¹⁰, Maria Rosa Caruso¹¹, Livia Garavelli¹², Simeone Andrucci¹³, Gianluca Vergine¹⁴, Nunzia Miglietti¹⁵, Elena Mancini¹⁶, Cristina Malaventura¹⁷, Antonio Percesepe¹⁸, Enrico Grosso¹⁹, Marco Materassi², Paola Romagnani^{1,2,20} and Sabrina Giglio^{1,3}

¹Department of Biomedical Experimental and Clinical Sciences "Mario Serio", University of Florence, Florence, Italy; ²Nephrology and Dialysis Unit, Meyer Children's University Hospital, Florence, Italy; ³Medical Genetics Unit, Meyer Children's University Hospital, Florence, Italy; ⁴Endocrinology Unit, Department of Health Sciences, University of Florence, Florence, Italy; ⁵Pediatric Nephrology Dialysis and Transplant Unit, Department of Pediatrics, University of Padua, Padua, Italy; ⁶Nephrology Dialysis, Transplant, Bambin Gesù Children's Hospital, Roma, Italy; ⁷Pediatric Nephrology and Dialysis Unit, Children's Hospital Giovanni XXIII, Bari, Italy; ⁸Medical Genetic Service, Department of Laboratory, S. Chiara Hospital, Trento, Italy; ⁹Division of Nephrology, Dialysis and Renal Transplantation, Niguarda Ca'Granda Hospital, Milan, Italy; ¹⁰Medical Genetics Unit, Department of Laboratory Medicine, Niguarda Ca'Granda Hospital, Milan, Italy; ¹¹Nephrology and Dialysis Unit, Papa Giovanni XXIII Hospital, Bergamo, Italy; ¹²Genetics Unit, Department of Obstetrics and Paediatrics, IRCCS S. Maria Nuova Hospital, Reggio Emilia, Italy; ¹³Department of Nephrology and Dialysis, Alessandro Manzoni Hospital, Lecco, Italy; ¹⁴Pediatric Unit, Ospedale degli Infermi, Rimini, Italy; ¹⁵Pediatric Unit, Azienda Ospedaliera Spedali Civili, Brescia, Italy; ¹⁶Division of Nephrology, Dialysis and Hypertension, Policlinico S. Orsola-Malpighi, Bologna, Italy; ¹⁷Department of Medical Sciences, Section of Pediatrics, University of Ferrara, Italy; ¹⁸Medical Genetics, Department of Clinical and Experimental Medicine, University Hospital of Parma, Italy; ¹⁹Department of Medical Sciences, University of Torino, Turin, Italy; and ²⁰Excellence Center DENOTHE, University of Florence, Florence, Italy

Primary distal renal tubular acidosis is a rare genetic disease. Mutations in *SLC4A1*, *ATP6V0A4*, and *ATP6V1B1* genes have been described as the cause of the disease, transmitted as either an autosomal dominant or recessive trait. Particular clinical features, such as sensorineural hearing loss, have been mainly described in association with mutations in one gene instead of the others. Nevertheless, the diagnosis of distal renal tubular acidosis is essentially based on clinical and laboratory findings, and the series of patients described so far are usually represented by small cohorts. Therefore, a strict genotype-phenotype correlation is still lacking, and questions about whether clinical and laboratory data should direct the genetic analysis remain open. Here, we applied next-generation sequencing in 89 patients with a clinical diagnosis of distal renal tubular acidosis, analyzing the prevalence of genetic defects in *SLC4A1*, *ATP6V0A4*, and *ATP6V1B1* genes and the clinical phenotype. A genetic cause was determined in 71.9% of cases. In our group of sporadic cases, clinical features, including sensorineural

hearing loss, are not specific indicators of the causal underlying gene. Mutations in the *ATP6V0A4* gene are quite as frequent as mutations in *ATP6V1B1* in patients with recessive disease. Chronic kidney disease was frequent in patients with a long history of the disease. Thus, our results suggest that when distal renal tubular acidosis is suspected, complete genetic testing could be considered, irrespective of the clinical phenotype of the patient.

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Renal tubular acidosis (RTA) is characterized by persistent, normal serum anion gap metabolic acidosis. Different types of RTA can be distinguished on the basis of clinical, pathophysiologic, and molecular criteria. Primary forms result from specific genetic defects in transporters/enzymes involved in renal bicarbonate (HCO_3^-) reabsorption or hydrogen (H^+) secretion and usually become clinically evident during infancy or early childhood.¹

Distal RTA (dRTA) is a rare genetic disorder in which the main clinical features are vomiting, diarrhea and/or constipation, loss of appetite, polydipsia and polyuria, nephrocalcinosis, nephrolithiasis, osteomalacia, and rickets. Most

Correspondence: Sabrina Giglio, Department of Clinical, Experimental, and Biomedical Sciences, Medical Genetics Unit, Meyer Children's University Hospital, Viale Pieraccini 24, 50139 Florence, Italy. E-mail: s.giglio@meyer.it; sabrinagiglio@unifi.it

²¹These authors contributed equally to this work.

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pediatric cases are primary, and patients are often affected by growth retardation caused by chronic metabolic acidosis unless alkali therapy is initiated early in life. In this disorder, the α -intercalated cells in the collecting duct are unable to secrete H^+ and to properly acidify urine or to reabsorb HCO_3^- .^{1,2}

dRTA can be transmitted as either an autosomal dominant (AD) or autosomal recessive (AR) trait.³ AD forms typically become clinically manifest in adolescence or adulthood and are usually caused by mutations in the *SLC4A1* gene, encoding the basolateral Cl^-/HCO_3^- exchanger.^{1,4,5} This gene has different promoter regions and can undergo alternative splicing, thus regulating the expression and sequence characteristics of the kidney and erythrocyte isoforms. Therefore, mutations in the *SLC4A1* gene can cause dRTA and/or hemolytic anemia with red cell morphology anomalies, also in a recessive manner.^{6,7}

AR dRTA is associated with mutations in *ATP6V0A4* and *ATP6V1B1* genes encoding for the A4 and B1 subunits of the apical H^+ -ATPase pump, respectively. Patients can present with early or absent/late sensorineural hearing loss (SNHL). Subjects without hearing defects usually carry mutations in the *ATP6V0A4* gene (except for some rare variants), whereas those with deafness have mutations in the *ATP6V1B1* gene.^{8–18}

The diagnosis of dRTA is based on clinical and laboratory features. However, a molecular diagnosis is of great importance in order to provide the patient and the family with adequate genetic counseling to better assess the patient's prognosis and to define genotype-phenotype correlations. Nevertheless, only a small series of patients with dRTA has been studied and genetically characterized so far.^{4–26} For this reason, a strict and reliable genotype-phenotype correlation is still lacking.

In this study, we applied a next-generation sequencing approach to 89 patients with a clinical diagnosis of dRTA.

The aim of this work was to analyze the prevalence of genetic defects in *SLC4A1*, *ATP6V0A4*, and *ATP6V1B1* genes and to assess the clinical phenotype of patients that are eventually typical of the different genetic forms of the disease.

RESULTS

Genetic analysis

A total of 89 patients with a reported clinical diagnosis of dRTA were analyzed (Supplementary Tables S1 and S2). Consanguinity was noted only in 4 families (cases 19, 30, 60, and 64). All the patients underwent genetic testing for *SLC4A1*, *ATP6V1B1*, and *ATP6V0A4* genes. Among 89 patients, 64 showed causative mutations (71.9%) in 1 of the 3 genes (Tables 1–3, Figure 1a). Mutations were distributed as follows (Figure 1a): 9 patients presented pathogenic variants in the *SLC4A1* gene (10.1%) (Table 1, cases 1–9), 30 patients had causative mutations in the *ATP6V0A4* gene (33.7%) (Table 2, cases 10–39), and 25 patients had mutations in the *ATP6V1B1* gene (28.0%) (Table 3, cases 42–66).

Among patients carrying pathogenic variants in the *SLC4A1* gene, 7 (6.7%) satisfied criteria for a molecular diagnosis of AD dRTA (Table 1, cases 1–7), whereas 2 (2.2%) were homozygous (Table 1, cases 8 and 9). All the mutations were missense, and 6 of them were *de novo*. All of these variants were already reported as pathogenic.^{5,7,27–29}

In the *ATP6V0A4* gene, we found 11 missense, 6 nonsense, 5 splicing, and 5 frameshift mutations and 1 nonstop change and 1 intragenic deletion (Table 2). In addition, 3 cases showed a triplet exon deletion. Mutations were detected in 30 cases, 17 of them in homozygosis and 14 in compound heterozygosis. In 20 cases, it was possible to assess the pattern of inheritance through the analysis of both parents, whereas in the remaining cases, blood samples from the parents were not available. In 5 cases (cases 13, 20, 30, 32, and 35), we found homozygous pathogenic variants not reported in the literature yet; 7 cases (cases 14, 17, 21, 25, 26,

Table 1 | Cases with mutations in the *SLC4A1* gene

Case	Pathogenic mutations	Mother	Father	Relatives	Ref.
1	c.[1765C>T][+]=] p.[Arg589Cys][+]=]	WT	WT	WT	Bruce et al. ⁵
2	c.[1937G>A][+]=] p.[Arg646Gln][+]=]	NA	NA	NA	Zelinski et al. ²⁷
3	c.[1766G>A][+]=] p.[Arg589His][+]=]	WT	WT	WT	Bruce et al. ⁵
4	c.[1765G>A][+]=] p.[Arg589Cys][+]=]	WT	WT	NA	Bruce et al. ⁵
5	c.[1825G>A][+]=] p.[Gly609Arg][+]=]	WT	WT	NA	Rungraj et al. ²⁸
6	c.[1765C>T][+]=] p.[Arg589Cys][+]=]	WT	WT	WT	Bruce et al. ⁵
7	c.[1766G>A][+]=] p.[Arg589His][+]=]	WT	WT	WT	Bruce et al. ⁵
8	c.[388G>A][+388G>A] p.[Gly130Arg][+][Gly130Arg]	c.[388G>A][+]=] p.[Gly130Arg][+]=]	c.[388G>A][+]=] p.[Gly130Arg][+]=]	NA	Inoue et al. ²⁹
9	c.[2102G>A][+2102G>A] p.[Gly701Asp][+][Gly701Asp]	c.[2102G>A][+]=] p.[Gly701Asp][+]=]	c.[2102G>A][+]=] p.[Gly701Asp][+]=]	NA	Tanphaichitr et al. ⁷

NA, not available; WT, wild type; [=], represents the wild-type allele, consistent with Human Genome Variation Society (HGVS) nomenclature.

Table 2 | Cases with mutations in *ATP6V0A4* gene

Case	Pathogenic mutations	Mother	Father	Relatives	Ref.
10	c.[2420G>A]+[2420G>A] p.[Arg807Gln]+[Arg807Gln]	c.[2420G>A]+[=] p.[Arg807Gln]+[=]	c.[2420G>A]+[=] p.[Arg807Gln]+[=]	NA	Stover <i>et al.</i> ¹²
11	c.[1185delC]+[2420G>A] p.[Tyr396Thrfs*12]+[Arg807Gln]	c.[2420G>A]+[=] p.[Arg807Gln]+[=]	c.[1185delC]+[=] p.[Tyr396Thrfs*12]+[=]	NA	Stover <i>et al.</i> ¹²
12	c.[414_417+10del14]+[1571C>T] p.[splicing]+[Pro524Leu]	c.[414_417+10del14]+[=] p.[splicing]+[Pro524Leu]	c.[1571C>T]+[=] p.[Pro524Leu]+[=]	NA	Carboni <i>et al.</i> ¹⁰ Smith <i>et al.</i> ¹¹
13	c.[2195T>C]+[2195T>C] p.[Leu732Pro]+[Leu732Pro]	c.[2195T>C]+[=] p.[Leu732Pro]+[=]	c.[2195T>C]+[=] p.[Leu732Pro]+[=]	c.[2195T>C]+[2195T>C] p.[Leu732Pro]+[Leu732Pro] Sister	Vargas-Poussou <i>et al.</i> ⁹
14	c.[2137delG]+[2335-2337del3] p.[Glu713Serfs*50]+[Phe779del]	c.[2137delG]+[=] p.[Glu713Serfs*50]+[=]	c.[2335-2337del3]+[=] p.[Phe779del]+[=]	NA	Stover <i>et al.</i> ¹²
15	c.[2332delG]+[2521T>C] p.[Val778Phefs*11]+[*841Glnnext*53]	NA	NA	NA	Stover <i>et al.</i> ¹²
16	c.[1185delC]+[2137delG] p.[Tyr396Thrfs*12]+[Glu713Serfs*50]	c.[1185delC]+[=] p.[Tyr396Thrfs*12]+[=]	c.[2137delG]+[=] p.[Glu713Serfs*50]+[=]	NA	Stover <i>et al.</i> ¹² Vargas-Poussou <i>et al.</i> ⁹
17	c.[1336_1338del3]+[1345C>T] p.[Phe446del]+[Arg449Cys]	c.[1336_1338del3]+[=] p.[Phe446del]+[=]	c.[1345C>T]+[=] p.[Arg449Cys]+[=]	c.[1345C>T]+[=] p.[Arg449Cys]+[=] Sister	Nagara <i>et al.</i> ³⁰
18	c.[710_712del3]+[710_712del3] p.[Lys237del]+[Lys237del]	c.[710_712del3]+[=] p.[Lys237del]+[=]	c.[710_712del3]+[=] p.[Lys237del]+[=]	NA	Stover <i>et al.</i> ¹²
19	c.[16C>T]+[16C>T] p.[Arg6*]+[Arg6*]	c.[16C>T]+[16C>T] p.[Arg6*]+[Arg6*]	c.[16C>T]+[16C>T] p.[Arg6*]+[Arg6*]	c.[16C>T]+[=] p.[Arg6*]+[=] Brother	Vargas-Poussou <i>et al.</i> ⁹
20	c.[2215G>C]+[2215G>C] p.[Ala739Pro]+[Ala739Pro]	NA	NA	NA	
21	c.[1185delC]+[1754_1781dup28] p.[Tyr396Thrfs*12]+[Ile594Metfs*18]	c.[1185delC]+[=] p.[Tyr396Thrfs*12]+[=]	c.[1754_1781dup28]+[=] p.[Ile594Metfs*18]+[=]	NA	Stover <i>et al.</i> ¹²
22	c.[1345C>T]+[1345C>T] p.[Arg449Cys]+[Arg449Cys]	NA	NA	NA	Nagara <i>et al.</i> ³⁰
23	c.[2257C>T]+[2257C>T] p.[Gln753*]+[Gln753*]	c.[2257C>T]+[=] p.[Gln753*]+[=]	c.[2257C>T]+[=] p.[Gln753*]+[=]	NA	Smith <i>et al.</i> ¹¹
24	c.[1342G>A]+[2426A>G] p.[Gly448Arg]+[Hys809Arg]	c.[2426A>G]+[=] p.[Hys809Arg]+[=]	c.[1342G>A]+[=] p.[Gly448Arg]+[=]	WT	
25	c.[1185delC]+[1572G>A] p.[Tyr396Thrfs*12]+[Pro252Pro]	c.[1185delC]+[=] p.[Tyr396Thrfs*12]+[=]	c.[1572G>A]+[=] c.[Pro252Pro]+[=]	NA	Stover <i>et al.</i> ¹²
26	c.[1572G>A]+[2257C>T] p.[Pro252Pro]+[Gln753*]	c.[1572G>A]+[=] p.[Pro252Pro]+[=]	c.[2257C>T]+[=] p.[Gln753*]+[=]	NA	Smith <i>et al.</i> ¹¹
27	c.[2257C>T]+[2257C>T] p.[Gln753*]+[Gln753*]	c.[2257C>T]+[=] p.[Gln753*]+[=]	NA	NA	Smith <i>et al.</i> ¹¹
28	c.[1478+1G>A]+[2190C>G] p.[splicing]+[Tyr730*]	c.[1478+G>A]+[=] p.[splicing]+[=]	c.[2190C>G]+[=] p.[Tyr739*]+[=]	c.[1478+1G>A]+[=] p.[splicing]+[=] Brother	Stover <i>et al.</i> ¹²
29	c.[1185delC]+[1185delC] p.[Tyr396Thrfs*12]+[Tyr396Thrfs*12]	c.[1185delC]+[=] p.[Tyr396Thrfs*12]+[=]	c.[1185delC]+[=] p.[Tyr396Thrfs*12]+[=]	NA	
30	c.[52C>T]+[52C>T] p.[Gln18*]+[Gln18*]	c.[52C>T]+[=] p.[Gln18*]+[=]	c.[52C>T]+[=] p.[Gln18*]+[=]	c.[52C>T]+[52C>T] p.[Gln18*]+[Gln18*] Brother	

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Table 2 | (Continued) Cases with mutations in *ATP6V0A4* gene

Case	Pathogenic mutations	Mother	Father	Relatives	Ref.
31	c.[1920_1921delAA]+[2137delG] p.[Ser641Phefs*17]+[Glu713Serfs*50]	NA	NA	NA	Vargas-Poussou <i>et al.</i> ⁹
32	c.[196+4A>G]+[196+4A>G] p.[splicing]+[splicing]	NA	NA	NA	
33	c.[1185delC]+[2137delG] p.[Tyr396Thrfs*12]+[Glu713Serfs*50]	c.[1185delC]+[=] p.[Tyr396Thrfs*12]+[=]	c.[2137delG]+[=] p.[Glu713Serfs*50]+[=]	c.[1185delC]+[2137delG] p.[Tyr396Thrfs*12]+[Glu713Serfs*50] Sister	Stover <i>et al.</i> ¹² Vargas-Poussou <i>et al.</i> ⁹
34	c.[292-1G>A]+[292-1G>A] p.[splicing]+[splicing]	c.[292-1G>A]+[=] p.[splicing]+[=]	c.[292-1G>A]+[=] p.[splicing]+[=]	NA	Smith <i>et al.</i> ¹¹
35	c.[2426A>C]+[2426A>C] p.[Hys809Arg]+[Hys809Arg]	NA	NA	NA	
36	c.[571C>T]+ del(7q34) (138,080,420-138,080,738) p.[R191*]+[exon 14 skipping]	NA	NA	NA	Vargas-Poussou <i>et al.</i> ⁹
37	c.[580C>T]+[=] p.[Arg194*]+[=] c.[2426A>G]+[2426A>G] p.[Hys809Arg]+[Hys809Arg]	NA	NA	NA	Stover <i>et al.</i> ¹²
38	c.[1561G>A]+[1561G>A] p.[Gly521Arg]+[Gly521Arg] c.[1888G>A]+[1888G>A] p.[Ala630Thr]+[Ala630Thr]	NA	NA	NA	
39	c.[1335C>G]+[1335C>G] p.[Phe445Leu]+[Phe445Leu] c.[1367G>A]+[1367G>A] p.[Gly456Asp]+[Gly456Asp]	c.[1335C>G]+[=] p.[Phe445Leu]+[=] c.[1367G>A]+[=] p.[Gly456Asp]+[=]	c.[1335C>G]+[=] p.[Phe445Leu]+[=] c.[1367G>A]+[=] p.[Gly456Asp]+[=]	NA NA	

NA, not available; WT, wild type; [=], represents the wild-type allele, consistent with HGVS nomenclature. Variants not reported in literature are highlighted in bold. For these variants, prediction of pathogenicity is reported in [Supplementary Table S3](#).

Table 3 | Cases with mutations in the *ATP6V1B1* gene

Case	Pathogenic mutations	Mother	Father	Relatives	Ref.
42	c.[242C>T]+[242C>T] p.[Leu81Pro]+[Leu81Pro]	c.[242C>T]+[=] p.[Leu81Pro]+[=]	c.[242C>T]+[=] p.[Leu81Pro]+[=]	NA	Karet <i>et al.</i> ⁸
43	c.[909+1G>T]+[1037C>G] p.[splicing]+[Pro346Arg]	c.[909+1G>T]+[=] p.[splicing]+[=]	c.[1037C>G]+[=] p.[Pro346Arg]+[=]	NA	Karet <i>et al.</i> ⁸
44	c.[242C>T]+[242C>T] p.[Leu81Pro]+[Leu81Pro]	c.[242C>T]+[=] p.[Leu81Pro]+[=]	c.[242C>T]+[=] p.[Leu81Pro]+[=]	NA	Karet <i>et al.</i> ⁸
45	c.[687+1G>T]+[687+1G>T] p.[splicing]+[splicing]	c.[687+1G>T]+[=] p.[splicing]+[=]	c.[687+1G>T]+[=] p.[splicing]+[=]	NA	Karet <i>et al.</i> ⁸
46	c.[484G>T]+[497delC] p.[Glu162*]+[Thr166Argfs*9]	c.[497delC] p.[Tr166Argfs*]+[=]	c.[484G>T]+[=] p.[Glu162*]+[=]	NA	Karet <i>et al.</i> ⁸
47	c.[242C>T]+[242C>T] p.[Leu81Pro]+[Leu81Pro]	NA	NA	NA	Karet <i>et al.</i> ⁸
48	c.[1155dupC]+[823A>C] p.[Ile386Hisfs*56]+[Thr275Pro]	c.[1155dupC]+[=] p.[Ile386Hisfs*56]+[=]	c.[823A>C]+[=] p.[Thr275Pro]+[=]	c.[1155dupC]+[823A>C] p.[Ile386Hisfs*56]+[Thr275Pro] Sister	Karet <i>et al.</i> ⁸
49	c.[242C>T]+[1007_1010del4] p.[Leu81Pro]+[Glu336Valfs*26]	c.[1006_1010del4]+[=] p.[Glu336Valfs*27]+[=]	c.[242C>T]+[=] p.[Leu81Pro]+[=]	c.[242C>T]+[=] p.[Leu81Pro]+[=] Sister	Karet <i>et al.</i> ⁸
50	c.[943C>T]+[1397C>A] p.[Arg315*]+[Ser466*]	NA	NA	NA	Karet <i>et al.</i> ⁸
51	c.[1037C>G]+[1037C>G] p.[Pro346Arg]+[Pro346Arg]	c.[1037C>G]+[=] p.[Pro346Arg]+[=]	NA	NA	Karet <i>et al.</i> ⁸
52	c.[1155dupC]+[1155dupC] p.[Ile386Hisfs*56]+[Ile386Hisfs*56]	c.[1155dupC]+[=] p.[Ile386Hisfs*56]+[=]	c.[1155dupC]+[=] p.[Ile386Hisfs*56]+[=]	NA	Karet <i>et al.</i> ⁸
53	c.[408delG]+[687+1G>T] p.[Pro137Glnfs*27]+[splicing]	c.[687+1G>T]+[=] p.[splicing]+[=]	c.[408delG]+[=] p.[Pro137Glnfs*27]+[=]	NA	Karet <i>et al.</i> ⁸
54	c.[1155dupC]+[1155dupC] p.[Ile386Hisfs*56]+[Ile386Hisfs*56]	c.[1155dupC]+[=] p.[Ile386Hisfs*56]+[=]	c.[1155dupC]+[=] p.[Ile386Hisfs*56]+[=]	c.[1155dupC]+[1155dupC] p.[Ile386Hisfs*56]+[Ile386Hisfs*56] Sister	Karet <i>et al.</i> ⁸
55	c.[539G>A]+[814G>C] p.[Gly180Asp]+[Ala272Pro]	c.[814G>C]+[=] p.[Ala272Pro]+[=]	NA	NA	
56	c.[1155dupC]+[1155dupC] p.[Ile386Hisfs*56]+[Ile386Hisfs*56]	c.[1155dupC]+[=] p.[Ile386Hisfs*56]+[=]	c.[1155dupC]+[=] p.[Ile386Hisfs*56]+[=]	c.[1155dupC]+[1155dupC] p.[Ile386Hisfs*56]+[Ile386Hisfs*56] Brother	Karet <i>et al.</i> ⁸
57	c.[1037C>G]+[1397C>A] p.[Pro346Arg]+[Ser466*]	c.[1037C>G]+[=] p.[Pro346Arg]+[=]	c.[1397C>A]+[=] p.[Ser466*]+[=]	NA	Karet <i>et al.</i> ⁸
58	c.[1155dupC]+[1155dupC] p.[Ile386Hisfs*6]+[Ile386Hisfs*56]	c.[1155dupC]+[=] p.[Ile386Hisfs*56]+[=]	c.[1155dupC]+[=] p.[Ile386Hisfs*56]+[=]	c.[1155dupC]+[1155dupC] p.[Ile386Hisfs*56]+[Ile386Hisfs*56] Brother	Karet <i>et al.</i> ⁸
59	c.[33dupG]+[1037C>G] p.[Leu12Alafs*5]+[Pro346Arg]	c.[33dupG]+[=] p.[Leu12Alafs*5]+[=]	c.[1037C>G]+[=] p.[Pro346Arg]+[=]	NA	Karet <i>et al.</i> ⁸
60	c.[1249-3C>G]+[1249-3C>G] p.[splicing]+[splicing]	c.[1249-3C>G]+[=] p.[splicing]+[=]	c.[1249-3C>G]+[=] p.[splicing]+[=]	c.[1249-3C>G]+[1249-3C>G] p.[splicing]+[splicing] Brother	
61	c.[1155dupC]+[1155dupC] p.[Ile386Hisfs*56]+[Ile386Hisfs*56]	c.[1155dupC]+[=] p.[Ile386Hisfs*56]+[=]	c.[1155dupC]+[=] p.[Ile386Hisfs*56]+[=]	NA	Karet <i>et al.</i> ⁸
62	c.[461C>G]+[497delC] p.[Pro154Arg]+[Thr166Argfs*9]	c.[461C>G]+[=] p.[Pro154Arg]+[=]	c.[497delC]+[=]p.[Thr166Argfs*9]+[=]	c.[461C>G]+[497delC] p.[Pro154Arg]+[Thr166Argfs*9] Brother	Karet <i>et al.</i> ⁸

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Table 3 | (Continued) Cases with mutations in the *ATP6V1B1* gene

Case	Pathogenic mutations	Mother	Father	Relatives	Ref.
63	c.[242C>T]+[687+1G>A] p.[Leu81Pro]+[splicing]	c.[242C>T]+[=] p.[Leu81Pro]+[=]	c.[687+1G>A]+[=] p.[splicing]+[=]	NA	Karet et al. ⁸
64	c.[175-1G>C]+[175-1G>C] p.[splicing]+[splicing]	c.[175-1G>C]+[=] p.[splicing]+[=]	c.[175-1G>C]+[=] p.[splicing]+[=]	NA	Vargas-Poussou et al. ⁹
65	c.[1155dupC]+[1155dupC] p.[Ile386Hisfs*56]+[Ile386Hisfs*56]	c.[1155dupC]+[=] p.[Ile386Hisfs*56]+[=]	c.[1155dupC]+[=] p.[Ile386Hisfs*56]+[=]	NA	Karet et al. ⁸
66	c.[1037C>G]+[1037C>G] p.[Pro346Arg]+[Pro346Arg]	c.[1037C>G]+[=] p.[Pro346Arg]+[=]	c.[1037C>G]+[=] p.[Pro346Arg]+[=]	NA	Karet et al. ⁸

NA, not available; WT, wild type; [=], represents the wild-type allele, consistent with HGVS nomenclature. Variants not reported in literature are highlighted in bold. For these variants, prediction of pathogenicity is reported in Supplementary Table S3.

31, 36) presented novel heterozygous mutations with another already described causative variant.^{9–12,30} Cases 24 and 28 showed new pathogenic variants in compound heterozygosity. Case 36 presented a missense mutation in the exon 8 and a novel intragenic deletion (including intron 13 and exon 14) in the other allele. This is a unique case with a genomic rearrangement detected by custom array-comparative genomic hybridization (a-CGH). Case 37 showed a novel homozygous mutation in exon 21 along with a heterozygous variant in exon 8, already characterized in recessive forms of dRTA.¹² In addition, in 2 patients we found 2 different homozygous mutations in the 15 and 17 (case 38) and 14 (case 39) exons.

In the *ATP6V1B1* gene, we found 6 missense, 3 nonsense, 5 splicing, and 5 frameshift mutations, either in homozygosity (14 cases) or compound heterozygosity (11 cases) (Table 3). We identified 12 novel variants: case 60 presented a novel homozygous splicing mutation, with the elimination of the acceptor site splice in exon 13, whereas case 55 showed 2 novel missense mutations in compound heterozygosity. In 21 cases, it was possible to assess the pattern of inheritance by the analysis of both parents.

Moreover, 7 additional patients (7.9%) were found to have genetic variants that did not satisfy criteria for a conclusive molecular diagnosis and were therefore classified as variants of unknown clinical significance (Table 4, Figure 1a).³¹ In particular, 5 patients presented heterozygous variants in the *ATP6V0A4* gene (Table 4, cases 40 and 41) or the *ATP6V1B1* gene (Table 4, cases 67–69), whereas 2 cases presented 2 different variants in a “digenic” pattern of inheritance, involving the *ATP6V0A4/ATP6V1B1* genes in 1 case (case 70) and the *ATP6V1B1/SLC4A1* genes in another case (case 71). In the latter case, both variants have not yet been reported in the literature. Interestingly, all of these patients presented with the classic clinical features of dRTA.

In addition, in the completely negative patients (18 patients) as well as in all of the *ATP6V0A4* and *ATP6V1B1* heterozygous cases (5 patients), the a-CGH allowed us to exclude any imbalance rearrangements inside the genes or in their flanking regions.

Clinical features and genotype-phenotype correlation

In the majority of patients included in this study, the clinical suspicion of dRTA arose because of failure to thrive (FTT) and vomiting, and the clinical diagnosis was confirmed with laboratory tests showing hyperchloremic metabolic acidosis with a simultaneous positive urinary anion gap and the inability to maximally acidify the urine (Supplementary Table S2).³² Biochemical data resulting from provocative tests were not available in our cohort. Although no significant difference was evident by analyzing the mean values of venous blood pH and urinary pH (Figure 2a,b) in patients with and without pathogenic mutations, levels of serum HCO₃⁻ and potassium were slightly different (Figure 2c,d, Student *t* test, *P* < 0.05), suggesting a more severe grade of metabolic acidosis in patients with mutations that is not fully reflected only by the mean of pH.

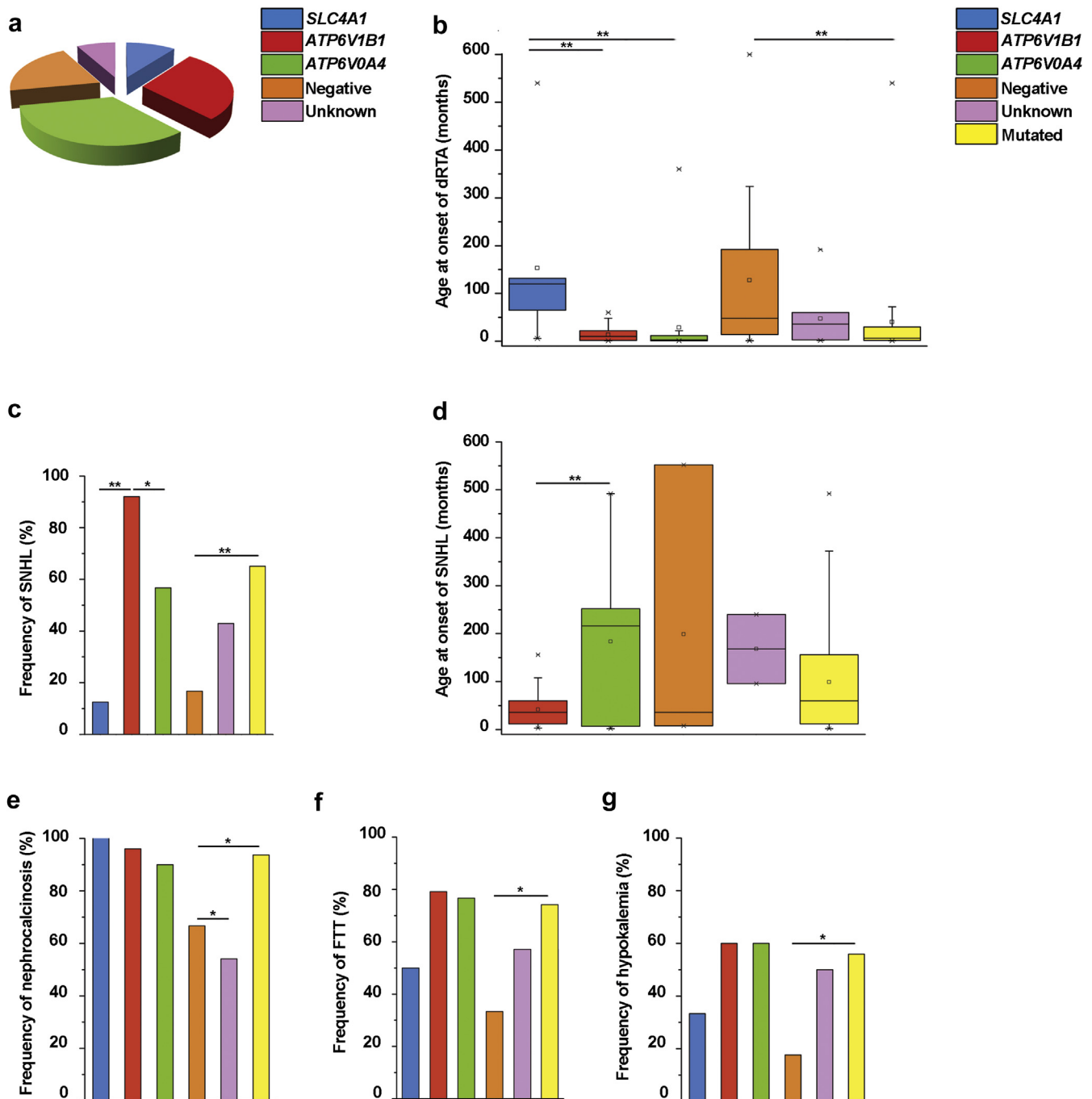


Figure 1 | Molecular characterization and clinical features of patients with distal renal tubular acidosis (dRTA). (a) Results of the genetic testing performed in 89 patients with a clinical diagnosis of dRTA. The frequency of mutations in *SLC4A1*, *ATP6V1B1*, and *ATP6V0A4* genes responsible for dRTA is reported. The frequency of negative cases and of variants of unknown clinical significance is also reported. (b) Age at clinical diagnosis. In each box, the bottom and top of the box are always the first and third quartiles, and the band inside the box is always the second quartile (the median). Squares inside each box represent the mean value for each group. The whiskers indicate variability outside the upper and lower quartiles, with the end of the whiskers representing 5th and 95th percentiles. (c) Frequency of sensorineural hearing loss (SNHL). (d) Age at onset of SNHL. In each box, the bottom and top of the box are always the first and third quartiles, and the band inside the box is always the second quartile (the median). Squares inside each box represent the mean value for each group. The whiskers indicate variability outside the upper and lower quartiles, with the end of the whiskers representing 5th and 95th percentiles. (e) Frequency of nephrocalcinosis. (f) Frequency of failure to thrive (FTT). (g) Frequency of hypokalemia. All the frequencies are reported as the percentage of patients presenting with a specific clinical feature among the total number of patients included in the study with available information. Throughout the figure, blue represents patients with pathogenic mutations in the *SLC4A1* gene (either autosomal dominant or autosomal recessive), red represents patients with pathogenic mutations in the *ATP6V1B1* gene (AR), green represents patients with pathogenic mutations in the *ATP6V0A4* gene (AR), orange represents patients without pathogenic mutations in the 3 genes analyzed, lilac represents patient carrying mutations that did not satisfy criteria for a molecular diagnosis (heterozygous variants in *ATP6V1B1* or *ATP6V0A4* genes or digenic inheritance; unknown), and yellow represents patients with pathogenic mutations in 1 of the 3 analyzed genes (mutated = *SLC4A1* + *ATP6V1B1* + *ATP6V0A4*). * $P < 0.05$; ** $P < 0.001$.

Table 4 | Cases with genetic variants that did not satisfy criteria for a molecular diagnosis

Patient	Pathogenic mutations	Mother	Father	Relatives	Ref.
<i>ATP6V0A4</i>					
40	c.[816+2T>C]+[=] p.[splicing]+[=]	NA	c.[816+2T>C]+[=] p.[splicing]+[=]	NA	Smith <i>et al.</i> ¹¹
41	c.[2035G>T]+[=] p.[Asp679Tyr]+[=]	NA	NA	NA	Vargas-Poussou <i>et al.</i> ⁹
<i>ATP6V1B1</i>					
67	c.[1181G>A]+[=] p.[Arg394Gln]+[=]	WT	WT	WT	Vargas-Poussou <i>et al.</i> ⁹
68	c.[1181G>A]+[=] p.[Arg394Gln]+[=]	WT	NA	NA	Vargas-Poussou <i>et al.</i> ⁹
69	c.[1037C>G]+[=] p.[Pro346Arg]+[=]	WT	[1037C>G]+[=] p.[Pro346Arg]+[=]	NA	Karet <i>et al.</i> ⁸
Digenic					
70	c.[687+1G>T]+[=] p.[splicing]+[=] <i>ATP6V1B1</i> c.[2035G>T]+[=] p.[Asp679Tyr] <i>ATP6V0A4</i>	WT	c.[687+1G>T]+[=] p.[splicing]+[=] <i>ATP6V1B1</i> c.[2035G>T]+[=] p.[Asp679Tyr] <i>ATP6V0A4</i>	NA	Karet <i>et al.</i> ⁸
71	c.[687G>A]+[=] p.[Gly228Gly]+[=] <i>ATP6V1B1</i> c.[2122C>]+[=] p.[Leu708Met]+[=] <i>SLC4A1</i>	c.[687G>A]+[=] p.[Gly228Gly]+[=] <i>ATP6V1B1</i> c.[2122C>A]+[=] p.[Leu708Met]+[=] <i>SLC4A1</i>	WT WT	c.[687G>A]+[=] p.[Gly228Gly]+[=] <i>ATP6V1B1</i> Brother [2122C>A]+[=] p.[Leu708Met]+[=] <i>SLC4A1</i> Brother	Vargas-Poussou <i>et al.</i> ⁹

NA, not available; WT, wild type; [=], wild-type allele, consistent with Human Genome Variation Society nomenclature.

Variants not reported in literature are highlighted in bold. For these variants, prediction of pathogenicity is reported in [Supplementary Table S3](#).

The male/female ratio of the subjects included in the study was 43/46 (48.3% male) ([Table 5](#)). The great majority of patients were from unrelated families of Caucasian origin. Only 15 patients had a different ethnicity (7 Africans, 6 Asian, and 2 South Americans) ([Supplementary Table S1](#)).

Age at diagnosis. With regard to the age at clinical diagnosis of dRTA, we found a mean age of 65.2 months in the group of patients with causative mutations ([Table 5](#), [Figure 1b](#)). Consistent with previous reports,⁶ the mean age at diagnosis was significantly older in patients with mutations in the *SLC4A1* gene (153.2 months; range, 6–540 months) compared with that of patients with mutations in the *ATP6V1B1* gene (13.9 months; range, 1–60 months; Mann-Whitney *U* test = 211.5, *P* < 0.001) or in the *ATP6V0A4*

gene (28.6 months; range, 1–360 months; Mann-Whitney *U* test = 243, *P* < 0.001).

Patients without mutations ([Supplementary Table S1](#), cases 72–89) had a mean age at clinical diagnosis of 131.1 months (range, 1–600 months) ([Table 5](#), [Figure 1b](#)). The age at diagnosis was significantly different in the group of patients with mutations compared with those without mutations (Mann-Whitney *U* test = 181.5, *P* < 0.05) ([Figure 1b](#)). On the contrary, no significant difference was found by comparing the group with mutations and the group of patients carrying variants of unknown clinical significance (Mann-Whitney *U* test = 166, not significant) ([Figure 1b](#)).

SNHL. Surprisingly, 1 case of SNHL was reported among patients with pathogenic variants in the *SLC4A1*

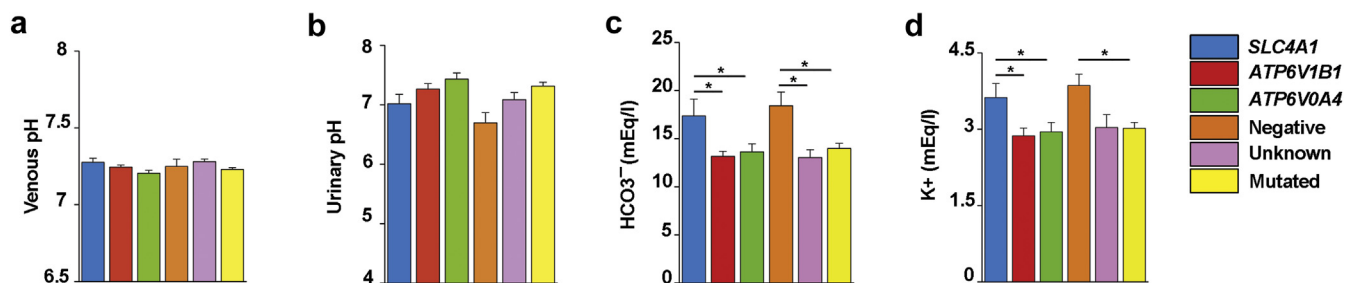


Figure 2 | Summary of data for laboratory findings for the patients included in the study. (a) Venous blood pH. (b) Urinary pH. (c) Serum HCO₃⁻ (mEq/l). (d) Serum potassium (mEq/l). Serum creatinine levels at onset (mg/dl). Serum creatinine levels at last follow-up (mg/dl). Throughout the figure, blue represents patients with pathogenic mutations in the *SLC4A1* gene, red represents patients with pathogenic mutations in the *ATP6V1B1* gene, green represents patients with pathogenic mutations in the *ATP6V0A4* gene, orange represents patients without pathogenic mutations in the 3 genes analyzed, lilac represents patients carrying mutations that did not satisfy criteria for a molecular diagnosis (unknown), and yellow represents patients with pathogenic mutations in 1 out the 3 analyzed genes (mutated = *SLC4A1* + *ATP6V1B1* + *ATP6V0A4*). **P* < 0.05. K⁺, serum potassium.

Table 5 | Clinical features of patients included in the study

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

CKD, chronic kidney disease (defined as estimated glomerular filtration rate <90 ml/min per 1.73 m²), dRTA, distal renal tubular acidosis; FTT, failure to thrive; M/F, male/female; SNHL, sensorineural hearing loss.

gene (Table 5, Figure 1c). In addition, 92% were reported to have hearing impairment in the group of patients carrying causative mutations in the *ATP6V1B1* gene (Table 5, Figure 1c). Moreover, 17 of 30 patients with pathogenic variants in the *ATP6V0A4* gene had SNHL, determining a prevalence of such a phenotypic feature as high as 56.7%. This frequency, although significantly lower than that found in patients with causative mutations in the *ATP6V1B1* gene ($\chi^2 = 6.89$, $P < 0.05$), was slightly higher than that reported in patients with mutations in the *ATP6V0A4* gene described so far.^{11,13,14} In patients with mutations in the *ATP6V1B1* gene, SNHL has an earlier clinical onset compared with patients carrying mutations in the *ATP6V0A4* gene (Mann-Whitney U test = 93, $P < 0.05$) (Table 5, Figure 1d). Interestingly enough, however, patients with mutations in the *ATP6V0A4* gene presented a wider range of clinical onset of SNHL (range, 2–552 months) (Figure 1e), encompassing infancy and early childhood, thus suggesting that an early onset of this clinical feature is not exclusive of *ATP6V1B1* gene variants.

In addition, SNHL was found in 3 of 18 patients (16.7%) negative at the genetic testing for the 3 genes (Table 5, Figure 1c); in this group, the age at onset was particularly variable (Table 5, Figure 1d). On the other hand, in patients carrying variants of unknown clinical significance, 42.9% had SNHL, a frequency not significantly different from that found in patients with mutations ($\chi^2 = 0.55$, not significant).

Nephrocalcinosis. On the basis of ultrasound renal scanning, nephrocalcinosis was found in 59 of 63 patients with mutations (93.6%) (Table 5, Figure 1e). No significant difference was found in the frequency of nephrocalcinosis in the 3 groups of patients with mutations (*SLC4A1*: 100%; *ATP6V0A4*: 90%; *ATP6V1B1*: 96%, all not significant). On the other hand, only 66.6% of patients with negative results on our genetic screening and 57.1% of patients with variants of unknown clinical significance presented this finding ($\chi^2 = 7.09$, $P < 0.05$ and $\chi^2 = 5.71$, $P < 0.05$ compared with patients with mutations, respectively).

FTT. FTT was reported as a common clinical sign at the onset of suspected dRTA (Supplementary Table S1). It was found in 4 of 8 patients with mutations in the *SLC4A1* gene (50.0%), in 19 of 24 with mutations in the *ATP6V1B1* gene (79.1%) and in 23 of 30 with mutations in the *ATP6V0A4*

gene (76.7%) (Table 5, Figure 1f). It was also present at a high frequency in patients with variants of unknown clinical significance (5/6 patients, 83.3%) (Table 5, Figure 1f).

Hypokalemia. Low potassium levels were found in 33 of 59 patients (55.9%) who satisfied criteria for a molecular diagnosis (Table 5, Figure 1g). In particular, hypokalemia was present in 3 of 9 patients (33.3%) with mutations in *SLC4A1*, 15 of 25 patients with mutations in *ATP6V1B1* (60.0%), and 15 of 25 patients with mutations in *ATP6V0A4* (60.0%). On the other hand, only 3 of 17 patients (17.6%) without mutations had low potassium levels. Interestingly enough, patients with mutations in genes encoding the A4 or B1 subunit of the H⁺-ATPase pump had a more severe hypokalemia compared with those with mutations in the *SLC4A1* gene, as previously reported (Figure 2d).³³ In any case, no clinical phenotype attributable to hypokalemia was reported in the clinical records. In addition, hypokalemia was milder in negative patients compared with those carrying pathogenic mutations.

Chronic kidney disease. Chronic kidney disease (CKD), defined as estimated glomerular filtration rate <90 ml/min per 1.73 m², was present in 31.3% of patients with pathogenic mutations (Table 5, Supplementary Tables S1 and S2). This frequency, although not significantly different compared with that found in patients with negative results on the genetic screening ($\chi^2 = 0$, $P < 0.05$) or in patients with mutations of unknown clinical significance ($\chi^2 = 0.49$, $P < 0.05$) is higher than that reported so far.^{34,35} Furthermore, CKD was evident in patients with long-term follow-up (at least 15 years) and always after puberty (Supplementary Figure S1). Indeed, by plotting the estimated glomerular filtration rate over time in a subset of patients with progressive worsening of renal function and pathogenic mutations in whom at least 3 measurements of serum creatinine were available, it was evident that the decline in kidney function started after pubertal growth spurt (Supplementary Figure S1).

DISCUSSION

The diagnosis of dRTA should be suspected whenever hyperchloremic metabolic acidosis is present without any obvious cause in the setting of relatively normal renal function.

In this study, we describe the largest dRTA cohort that has been genetically studied to date, finding a close correlation

between the clinical phenotype and the results of genetic testing. This approach allowed us to make some unexpected observations with important implications for the clinical management of patients affected by this disease: (i) most cases of dRTA are “sporadic” (>70%), although genetically transmitted, deriving from homozygous or compound heterozygous mutations, with a single family member affected; (ii) mutations in the *ATP6V0A4* gene are quite as frequent as mutations in the *ATP6V1B1* gene in patients with AR dRTA; (iii) in contrast to previous observations, the association of dRTA with early SNHL is not an absolute indicator of the underlying causal gene; (iv) CKD is more frequent than reported thus far and can occur in patients with a long history of the disease.

Most studies published to date described affected individuals grouped within families. Indeed, this disease is usually considered to be more prevalent in populations with a high rate of consanguinity, and generally the affected subjects present homozygous mutations. In this study, we showed that the large majority of cases occur in unrelated families, suggesting that a genetic form of dRTA should be suspected even in the absence of a familial history of the disease or consanguinity. Consistently, the cases reported in this study showed causative mutations transmitted in compound heterozygosity.

Although data on the real prevalence of the disease are still lacking, dRTA is considered to be extremely rare and can be transmitted with in an AD/AR manner, with or without deafness.²¹ The dominant form of the disease typically presents in adolescence or adulthood with a mild clinical phenotype and is associated with mutations in the basolateral $\text{Cl}^-/\text{HCO}_3^-$ exchanger encoded by the *SLC4A1* gene. Among the patients with mutations in the *SLC4A1* gene (Table 1), we detected a total of 6 variants. In some of these cases, we were able to highlight the presence of pathogenic variants of this gene also in childhood, although a more advanced age at onset of the clinical symptoms is usually reported. However, recessive variants occur in infancy or early childhood, as observed in our homozygous cases. The recessive mutations in the *SLC4A1* gene are associated with dRTA together with spherocytosis⁷ or, more frequently, with spherocytosis without renal involvement.²⁹ Consistent with already published data, the clinical and biochemical phenotype of patients with mutations in the *SLC4A1* gene is usually milder compared with that of patients with mutations in other causative genes. In particular, SNHL is not usually present, according to the lack of expression of the *SLC4A1* gene in the inner ear. Nonetheless, case 8 surprisingly had SNHL diagnosed at 12 years of age. This is the first report of these features in patients with mutations of this gene. It remains unclear whether biallelic mutations could cause deafness or because hearing loss is very common in the population, this trait can be attributed to pathogenic variants in other genes causative of SNHL.

The AR forms of dRTA have been associated with mutations in the *ATP6V1B1* gene or the *ATP6V0A4* gene.^{8–18,20}

In our cohort, mutations in the *ATP6V0A4* gene were quite as frequent as mutations in the *ATP6V1B1* gene. This is in contrast with what was previously reported because the frequency of mutations in the *ATP6V0A4* gene was about 2 times higher than that of mutations in the *ATP6V1B1* gene.^{8,11–16,19,30}

In addition, it has been typically believed that loss of function mutations in the *ATP6V1B1* gene caused dRTA with SNHL, whereas mutations in the *ATP6V0A4* gene were responsible for cases without SNHL or with hearing loss occurring later.^{11–18}

However, in our previous work, we considered the question of whether mutations in the *ATP6V0A4* gene could be responsible for early deafness, demonstrating that an enlarged vestibular aqueduct is associated with the precocity and severity of SNHL.¹⁹ Here we show that the association with SNHL is not an absolute indicator of the causal underlying gene, corroborating previous reported data and demonstrating the advantage of the next-generation sequencing approach instead of the selected Sanger phenotype-genotype sequencing approach, as described in previous reports.^{31,36} Our data suggest that even though SNHL is more frequent and has an earlier clinical onset in patients with mutations of *ATP6V1B1* gene, it does not represent a fully reliable clinical parameter for the identification of the gene involved. Indeed, the presence of early SNHL could at most allow us to distinguish patients with mutations in *ATP6V1B1* or *ATP6V0A4* from those with mutations in *SLC4A1*. Conversely, in infants with dRTA and SNHL, both *ATP6V1B1* and *ATP6V0A4* should be sequenced.

We also detected a large number of newly reported pathogenic variants, most of which were in the *ATP6V0A4* gene ($N = 18$) rather than the *ATP6V1B1* gene ($N = 12$). The missense mutations were probably pathogenic on the basis of the prioritization algorithm for newly identified variants that we and others previously applied (Supplementary Table S3).^{31,35} We evaluated the splice site variants using the program BDGP (Berkeley Drosophila Genome Project) for the *in silico* prediction. Unfortunately, the A4 and B1 subunits of the H⁺ATPase pump are not expressed in the blood circulating cells, making the study of the pathogenicity of these variants through the RNA expression impossible. Moreover, in case 36, showing the classic dRTA phenotype, we were able to identify an intragenic deletion with skipping of exon 14, associated with an already described mutation in compound heterozygous manner.⁹ This is the unique genomic rearrangement revealed by custom a-CGH carried out in all those cases that resulted negative or/and with a single heterozygous variant by sequencing analysis, in which it was necessary to find a pathogenic variant in the second allele.

The clinical significance of some genetic findings remains to be clarified. Cases 70 and 71 (Table 4) showed digenic heterozygous variants in *ATP6V0A4/ATP6V1B1* genes (Case 70) or in *ATP6V1B1/SLC4A1* genes (Case 71). These “double mutations” had both been inherited from the same parent who, however, did not display the classic clinical phenotype of

dRTA. This inconsistency in the phenotype could be explained by an incomplete penetrance in the relatives or by the presence of protective or modifier genes.

Moreover, cases 40 and 41 and 67 through 69 (Table 4) showed only heterozygous mutations in *ATP6V0A4* or *ATP6V1B1* genes associated with a classic dRTA phenotype. Imai *et al.*³⁷ recently described an adult patient with dRTA caused by a novel heterogeneous mutation of the A4 subunit of vacuolar H⁺-ATPase and a late clinical onset of the disease. Our cases presented early-onset dRTA, and the parents with mutations did not show any signs of disease. We can assume that it is possible that other genes expressed in the distal tubule or in other tubular segments and still unidentified are mutated.

Similarly, in patients who have a clinical phenotype consistent with dRTA and who were negative for the 3 known causative genes, it is possible that other genes potentially expressed in other tubule regions mimic the same clinical picture. In only 1 case, the clinical evaluation demonstrated the presence of Sjögren syndrome symptoms (case 89).

In 3 cases (cases 32, 41, and 68), medullary sponge kidney was diagnosed with i.v. urography (Supplementary Table S1). In particular, in case 32, we found a homozygous mutation in the *ATP6V0A4* gene, whereas in cases 41 and 68, genetic analysis revealed the patients to be carriers for heterozygous mutations in the *ATP6V0A4* and *ATP6V1B1* genes, respectively. The association between primary classic dRTA and medullary sponge kidney has already been described.^{10,38} Interestingly, the H⁺-ATPase pump is expressed in the α -intercalated cells of the late distal tubules and cortical collecting ducts, the same anatomic regions involved in medullary sponge kidney.¹⁰ However, the exact pathogenic relationship between dRTA and medullary sponge kidney is still lacking and needs to be clarified.

In conclusion, by analyzing the available clinical, laboratory, and instrumental data of 89 patients suspected of having dRTA, we can conclude that SNHL, nephrocalcinosis, FTT, and age at diagnosis actually allow patients with pathogenic mutations in 1 of the 3 causative genes to be differentiated from patients who are negative, thus suggesting that the initial clinical diagnosis of dRTA in the latter group should be reconsidered. Indeed, negative patients showed a less severe degree of metabolic acidosis and hypokalemia that, although clinically negligible, could be suggestive of a different pathogenesis. On the other hand, patients carrying variants of unknown clinical significance were not statistically different from those with pathogenic mutations for the majority of the clinical parameters, indicating that the variants identified in the first group are probably not insignificant in determining the clinical phenotype of patients.

In addition, the only parameters that presented a statistically significant difference in the 3 groups of mutated patients were, not surprisingly, the age at onset and the presence of SNHL that allowed us to distinguish patients with mutations in the *SLC4A1* gene from those with mutations in the *ATP6V1B1* or *ATP6V0A4* genes. Indeed, this difference has

already been reported.^{6,8,9,11–16,19,30} However, although patients with mutations in the *ATP6V1B1* gene had a higher prevalence and an earlier clinical onset of SNHL compared with patients carrying pathogenic variants of *ATP6V0A4*, SNHL should perhaps not be considered as an exclusive feature of the former group of patients. Our data suggest that these 2 genes must both be analyzed when dRTA is suspected, especially if SNHL is present and the patient is young.

Finally, we found a surprisingly high frequency of CKD in patients carrying pathogenic mutations. This is in contrast to what is reported in the literature.^{34,35} CKD occurs in patients with a long history of the disease and could be explained by the combination of nephrocalcinosis and persistent hypokalemia, leading to progressive tubulointerstitial damage, or by kidney damage following repeated episodes of dehydration and acute kidney injury. Interestingly enough, in our patients, CKD never occurred before adolescence and a pubertal growth spurt, probably because of compensatory hyperfiltration of functioning nephrons during childhood. This observation deserves particular attention because it suggests that, in the absence of previous clinical records, the diagnosis of dRTA could be missed in young adults presenting with moderately elevated serum creatinine levels and metabolic acidosis, as previously anecdotally reported.³⁹ Moreover, it raises the question on the “benignity” of the disease. Obviously, these data need to be confirmed in larger cohorts of patients with dRTA.

Taken together, these results allow us to conclude that in the case of clinically suspected dRTA (non-anion gap metabolic acidosis with inappropriate high urinary pH), the presence of accompanying clinical and laboratory features such as SNHL, nephrocalcinosis, and FTT and a less severe degree of metabolic acidosis and hypokalemia suggests that genetic testing be performed. This testing should always include the analysis of all 3 genes, unless a clear AD pattern of inheritance is reported.

METHODS

Patients

All the patients with dRTA (non-anion gap metabolic acidosis and the inability to maximally acidify the urine) and referred to Meyer Children's Hospital of Florence from 2011 to 2015 in need of a molecular diagnosis of the disease were included in the study. A total of 89 patients were recruited.

Demographic, clinical, laboratory, and instrumental data were collected retrospectively and are reported in Supplementary Tables S1 and S2. We tested patients for secondary forms of dRTA whenever the genetic sequencing failed to provide a molecular diagnosis of the disease. The estimated glomerular filtration rate was calculated using the Schwartz equation or MDRD equation, owing to the age of patients. The estimated glomerular filtration rate was used to assess the presence of CKD.

Parents and/or relatives were either included before this study or asked to participate after the identification of potentially causative variants in the proband. The study protocol was approved by the local Ethics Committee of Meyer Children's University Hospital, and informed written consent was obtained from the parents or legal

guardians of each study participant. The DNA samples were made anonymous by use of an alphanumeric code.

DNA extraction

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

Amplicon library preparation

To analyze sequence variations in *SLC4A1* (NM_000342), *ATP6V0A4* (NM_020632), and *ATP6V1B1* (NM_001692) genes, we used a strategy based on the locus-specific amplification of genomic DNA for each amplicon separately, followed by Roche 454 sequencing. Fusion primers were designed to generate tiled amplicons ranging in size from 300- to 400-bp segments (http://454.com/downloads/my454/documentation/gs-junior-plus/454SeqSys_Amplicon-Library-Prep-MM_Apr2014.pdf). At the 5' end, fusion primers contained a MID sequence that is a nucleotide tag that identifies the different samples. The MID was selected from a list provided by Roche (Supplementary Tables 4A and S4B). Thermal cycling was performed using the following profile: 1 cycle at 95°C for 5 minutes followed by 30 cycles at 95°C for 30 seconds, at the specific annealing temperature for 30 seconds, at 72°C/30 seconds, followed by a final extension step at 72°C for 7 minutes.

GS Junior Sequencing

The amplicon polymerase chain reaction-derived fragments were annealed to carrier beads and clonally amplified by emulsion polymerase chain reaction. Emulsion polymerase chain reaction and sequencing were performed according to the manufacturer's (Roche) protocol (<http://www.roche.it/home/roche-in-italia/diagnostics/prodotti/next-generation-seq.html>).

Data analysis

Data analysis was performed using Roche software. Image acquisition, processing, and signal processing were conducted during the run. A post-run analysis was performed using version 2.9 of GS Amplicon Variant Analyzer (<http://www.roche.it/home/roche-in-italia/diagnostics/prodotti/next-generation-seq.html>). The Amplicon Variant Analyzer application computes the alignment of reads from Amplicon libraries obtained on the GS Junior and identifies differences between the reads and a reference sequence. In this study, amplicon nucleotide sequence reads were aligned to the hg19 assembly genomic sequence of *SLC4A1*, *ATP6V0A4*, and *ATP6V1B1* genes. The Amplicon Variant Analyzer software identifies all nucleotide variants and provides read counts and frequencies (data available on request).

Variants prioritization

In order to identify genetic variants with a possible pathogenic significance, the algorithm for variant prioritization previously described was used.³¹ Variants already described were considered as pathogenic, in accordance with the pattern of inheritance. *In silico* analysis was performed using Polyphen-2 (<http://genetics.bwh.harvard.edu/pph2/>), SIFT (<http://sift.jcvi.org/>), Mutation Taster (<http://www.mutationtaster.org/>), or BDGP splice site prediction (http://www.fruitfly.org/seq_tools/splice.html) prediction tools (Supplementary Table S2).

Sanger sequencing

Potentially pathogenic variants detected by GS junior analyzer were confirmed by Sanger sequencing. Parents and relatives, whenever available, were also analyzed to determine the pattern of inheritance.

a-CGH

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

Statistical analysis

Statistical analysis was performed using SPSS software (SPSS, Inc., Evanston, IL). Frequencies between groups were compared by a χ^2 test, applying Yates' correction when appropriate. Between-group comparisons were made by using the Student *t* test for unpaired data or Mann-Whitney *U* test owing to normal or nonparametric distribution. A *P* value <0.05 was considered statistically significant.

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Plot of the estimated glomerular filtration rate over time in a subset of patients with pathogenic mutations.

Table S1. Clinical features of patients with distal renal tubular acidosis.

Table S2. Biochemical findings at baseline and serum creatinine levels at last follow-up in patients with distal renal tubular acidosis.

Table S3. *In silico* analysis of pathogenicity for missense variants not already described.

Table S4A. Design of fusion primer used for amplification of *SLC4A1*, *ATP6V0A4*, and *ATP6V1B1* genes with the GS Junior 454 system. Primer pairs were designed using MIDs. Complete sequences of forward and reverse primers for each genes are shown for *MID13* (in red and lilac).

Table S4B. Fusion primer design for screening with the GS Junior 454 system. The primers consist of 3 parts: the GS Junior 454 adaptor, containing the sequencing primers A and B, and the sequencing key "TCAG" (in blue), followed by a multiplex identifier (MID) tag that varies between samples. Primer pairs were designed using MIDs 1 through 14 and generating an amplicon of 400 b. Supplementary material is linked to the online version of the paper at www.kidney-international.org.

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