

Comprehensive Genetic Analysis Reveals Complexity of Monogenic Urinary Stone Disease



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Introduction: Because of phenotypic overlap between monogenic urinary stone diseases (USD), gene-specific analyses can result in missed diagnoses. We used targeted next generation sequencing (tNGS), including known and candidate monogenic USD genes, to analyze suspected primary hyperoxaluria (PH) or Dent disease (DD) patients genetically unresolved (negative; N) after Sanger analysis of the known genes. Cohorts consisted of 285 PH (PHN) and 59 DD (DDN) families.

Methods: Variants were assessed using disease-specific and population databases plus variant assessment tools and categorized using the American College of Medical Genetics (ACMG) guidelines. Prior Sanger analysis identified 47 novel PH or DD gene pathogenic variants.

Results: Screening by tNGS revealed pathogenic variants in 14 known monogenic USD genes, accounting for 45 families (13.1%), 27 biallelic and 18 monoallelic, including 1 family with a copy number variant (CNV). Recurrent genes included the following: *SLC34A3* (n = 13), *CLDN16* (n = 8), *CYP24A1* (n = 4), *SLC34A1* (n = 3), *SLC4A1* (n = 3), *APRT* (n = 2), *CLDN19* (n = 2), *HNF4A1* (n = 2), and *KCNJ1* (n = 2), whereas *ATP6V1B1*, *CASR*, and *SLC12A1* and missed CNVs in the PH genes *AGXT* and *GRHPR* accounted for 1 pedigree each. Of the 48 defined pathogenic variants, 27.1% were truncating and 39.6% were novel. Most patients were diagnosed before 18 years of age (76.1%), and 70.3% of biallelic patients were homozygous, mainly from consanguineous families.

Conclusion: Overall, in patients suspected of DD or PH, 23.9% and 7.3% of cases, respectively, were caused by pathogenic variants in other genes. This study shows the value of a tNGS screening approach to increase the diagnosis of monogenic USD, which can optimize therapies and facilitate enrollment in clinical trials.

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KEYWORDS: Dent disease; kidney stones; molecular genetics; monogenic; primary hyperoxaluria

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Urinary stone disease (USD) has an estimated worldwide prevalence of ~12%^{1–3} and is asso-

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ciated with significant morbidity including pain, hospitalizations, and surgical procedures, plus economic costs of the medical procedures and lost work time.⁴ Nephrocalcinosis (NC) is less prevalent than USD but is often associated with severe forms of USD and chronic kidney disease (CKD). Recurrence is seen in over 50% of USD patients⁵ and can be associated with progressive CKD.¹ Diet, water intake, and environment

are factors likely influencing stone formation, and changes in diet/lifestyle may have contributed to recent increases in USD prevalence in Western countries.⁶ However, as up to 50% of USD patients have an affected first-degree relative,^{2,3,7} and genome-wide association studies (GWAS) have linked multiple variants with USD,^{8,9} genetic risk factors are also important. Indeed, ~40 urinary stone and/or NC diseases are monogenic disorders.^{7,10} Recently, next generation sequencing (NGS) approaches, including targeted gene panels (tNGS) and whole exome sequencing (WES), within clinical nephrology have revealed the importance of monogenic disease as a cause of CKD and end-stage kidney disease (ESKD).¹¹ Application of tNGS using a panel of 30 known USD/NC causing genes within a stone-forming population determined that 11.4% of adults and 20.8% of the pediatric population had a likely monogenic cause.¹² Among USD patients presenting before 25 years of age, WES identified the likely causative gene in 15 of 51 cases (29.4%).¹³

Monogenic USD/NC tends to be more severe than sporadic USD, with earlier diagnosis and onset of symptoms as well as greater risk of CKD.⁷ Genetic screening is important because the clinical phenotype may not identify the precise cause of the disease, especially if only limited biochemical studies are conducted. Expedient diagnosis has important implications for early initiation of treatment of the disease. Increasingly, clinical trials based on a specific genetic and even allelic type are underway, with new treatments for specific monogenic USD/NC diseases rapidly emerging.^{14–16} A definitive genetic diagnosis can facilitate identification of other at-risk family members and potentially influence family planning decisions. Dominant (monoallelic) and X-linked causes of USD/NC have been identified; however, the majority of families have recessive (biallelic) inheritance, with enrichment in populations with a high level of consanguinity, which are often medically underserved.^{17,18} Although there is great promise for improved patient care following more extensive genetic screening, the importance of carefully evaluating of results in light of the particular disorder and patient phenotype, together with application of rigorous guidelines to evaluate genetic variants, are essential,¹⁹ because a misdiagnosis resulting in inappropriate treatment can be even more damaging than no diagnosis.

Two well-characterized monogenic causes of USD and NC are primary hyperoxaluria (PH) and Dent disease (DD). Primary hyperoxaluria is a disorder of hepatic glyoxylate metabolism characterized by oxalate overproduction that results in urinary calcium oxalate supersaturation and recurrent USD and/or NC, and frequently leads to loss of kidney function that can

progress to ESKD.^{20,21} Biallelic pathogenic variants to 3 separate genes cause the known types of PH: *AGXT* (PH1), *GRHPR* (PH2), and *HOGAI* (PH3).^{22–26} Dent disease is an X-linked recessive disease characterized by low-molecular-weight proteinuria (LMWP), hypercalciuria, and NC that may also cause ESKD. The 2 known DD genes are *CLCN5* (DD1) and *OCRL* (DD2).^{27–30} Here we used tNGS to rescreen a cohort of patients clinically suspected of having PH or DD but unresolved from targeted Sanger analysis. This study revealed significant phenotypic overlap between monogenic USDs and, hence, illustrated the value of a broad-based screening approach when a monogenic USD is suspected. In addition, careful variant evaluation also identified cases in which multiple genetic factors may contribute to the phenotype.

MATERIALS AND METHODS

Recruitment

The Rare Kidney Stone Consortium (RKSC) seeks to better understand hereditary forms of USD/NC.³¹ All patients provided signed consent for enrollment in the study protocol, which was approved by the relevant institutional review boards or ethics committees. The institutional review board protocol allowed recruitment and broad genomic analysis of patients suspected of monogenic USD/NC. The RKSC collaborators and the study coordinator team identified and consented patients and family members at collaborating RKSC sites worldwide. Patients in the current cohort were identified because of a suspicion of PH or DD, including the presence of USD and/or NC, often presenting before 18 years of age, and/or with CKD; however, because recessive (PH) and X-linked (DD) diseases were the focus, we did not require a positive family history. Patients with suspected PH often had evidence of hyperoxaluria and/or severe calcium oxalate stone disease, whereas LMWP and an apparent X-linked inheritance pattern suggested DD. However, detailed biochemical data were not always available, and investigators erred on the side of sensitivity rather than specificity when deciding whether to proceed with genetic testing. A blood sample was collected, and DNA was isolated, evaluated and quantified with the Trinean platform and stored at the Mayo Clinic Biospecimens Accessioning and Processing Core.

Sanger Screening of PH and DD Patients

This cohort was previously screened by Sanger sequencing of all coding exons for either the 3 PH genes (*AGXT*, NM_000030.2; *GRHPR*, NM_012203.1; *HOGAI*, NM_138413.3) or 2 DD genes (*CLCN5*, NM_000084.2; *OCRL*, NM_000276), depending on clinical suspicion.²⁶ All Sanger chromatograms were

analyzed using Mutation Surveyor (version 4.06; Soft-Genetics, State City, PA), and identified variants were categorized for pathogenicity using the American College of Medical Genetics and Genomics (ACMG) guidelines.¹⁹

NGS Library Generation and Sequencing

Next generation sequencing was performed with tNGS panels of 90 genes (coding region 171 kb, total captured region 485 kb) or 102 genes (coding region 211 kb; total captured region 560 kb) containing known monogenic USD/NC genes, plus candidate genes important for calcium metabolism or urinary components that contribute to lithogenicity, including oxalate, citrate, uric acid, or pH (Table S1). Our method for tNGS and variant evaluation has previously been described^{32,33} and is provided in detail in the [Supplementary Methods](#).

Distribution of Results

Research screening results were reported back to the referring physician, with the requirement of confirmation in a CLIA-approved laboratory before being used clinically.

RESULTS

Genetic Screening

Prior focused Sanger screening of 703 families suspected of PH determined that 268 were PH1, 47 PH2, and 52 PH3 (73.0%, 12.8%, and 14.2% of resolved families, respectively), a subset of which have been published,^{25,26,34,35} whereas 336 (47.8% of the total) were genetically negative (PHN) (Figure 1a). Among the 111 families suspected of DD, 44 had DD1 and 7 DD2 (86.3% and 13.7% of resolved, respectively), whereas 60 (54.1% of the total) remained unresolved (DDN). Table S2 contains details of the novel, unpublished Sanger-detected PH and DD gene variants.

The focus of the current study was the 285 PHN and 59 DDN families with available DNA (Figure 1a). These PHN and DDN families were rescreened with a tNGS panel of 90 ($n = 279$) or 102 ($n = 65$) known or candidate USD/NC genes (Table S1) to determine whether other monogenic USD genes might account for their phenotype. A rigorous evaluation of variants using ACMG guidelines was applied to detected variants (see [Supplementary Methods](#) for details) to carefully identify likely monogenic subjects. From this analysis, a likely cause of the disease due to variants in known monogenic USD genes was identified in 45 families (13.1%), 29 PHN (10.2%) and 16 DDN (27.1%) (see Figure 1b and Table 1 for genetic and clinical details). Of the resolved cases, 27 (60.0%) had biallelic disease and 18 (40.0%) had monoallelic disease, with 14

different genes implicated (Figure 1c). Of the 48 defined pathogenic variants, 19 (39.6%) were novel and 13 (27.1%) were truncating (see Table 2^{36–53} for details of scoring and ACMG guidelines evaluation).

Primary Hyperoxaluria

Two PHN families were found in fact to have PH using the tNGS panel reanalysis because CNVs were missed by Sanger screening. In PHN244, a large deletion from 5' of *AGXT* to *IVS2* was detected in homozygosity by CNV analysis of the NGS and confirmed by MLPA, that was also present in heterozygosity in both parents (Tables 1, 2). In the second family, PH2-6, previous Sanger analysis detected a 2-bp deletion on 1 *GRHPR* allele, but the reanalysis also detected the second likely pathogenic variant, a large deletion of exons 3 to 5 by CNV analysis of the NGS.

Familial Hypomagnesemia With Hypercalciuria and NC

Claudins 16 and 19 (encoded by *CLDN16* and *CLDN19*) regulate calcium and magnesium transport, and biallelic pathogenic variants are associated with familial hypomagnesemia with hypercalciuria and NC (FHHNC).^{38,54} The most common biallelically mutated gene in our cohort was *CLDN16*, found in 8 pedigrees, including 7 from the PHN cohort (Figure 1; Tables 1, 2). Seven different pathogenic variants were detected, 3 of which were novel. Interestingly, all 8 cases were homozygous, with consanguinity known or suspected in each family. All the subjects were diagnosed during childhood; 6 had NC, 4 had USD, and 4 experienced ESKD. Biallelic variants were also detected in a second claudin gene, *CLDN19*, in 2 male patients. Both patients were homozygous, 1 patient for a novel variant; both had NC, and 1 patient experienced ESKD.

Hypophosphatemic/Hypercalciuric Stone Formation With Bone Defects

Loss of the proximal tubular sodium-dependent phosphate transport proteins 2A (NaPi-IIa, encoded by *SLC34A1*) or 2C (NaPi-IIc, encoded by *SLC34A3*) results in reduced renal Pi reabsorption, and biallelic pathogenic variants have been associated with hypophosphatemia and hypercalciuria.^{46,47,55} Reports also suggest that monoallelic variants at these loci are associated with NC and USD, and GWAS have implicated *SLC34A1* variants in common USD.^{8,56–58} In this cohort, biallelic pathogenic variants to *SLC34A3* (4 families) or *SLC34A1* (1 family) were identified. All 4 *SLC34A3* patients were identified in the DDN cohort; 1 was homozygous, 2 alleles were novel, and in 2 families 3 alleles that may be significant were detected (Tables 1 and 2; Figure 2a, b⁴⁷). Atypical splicing was also

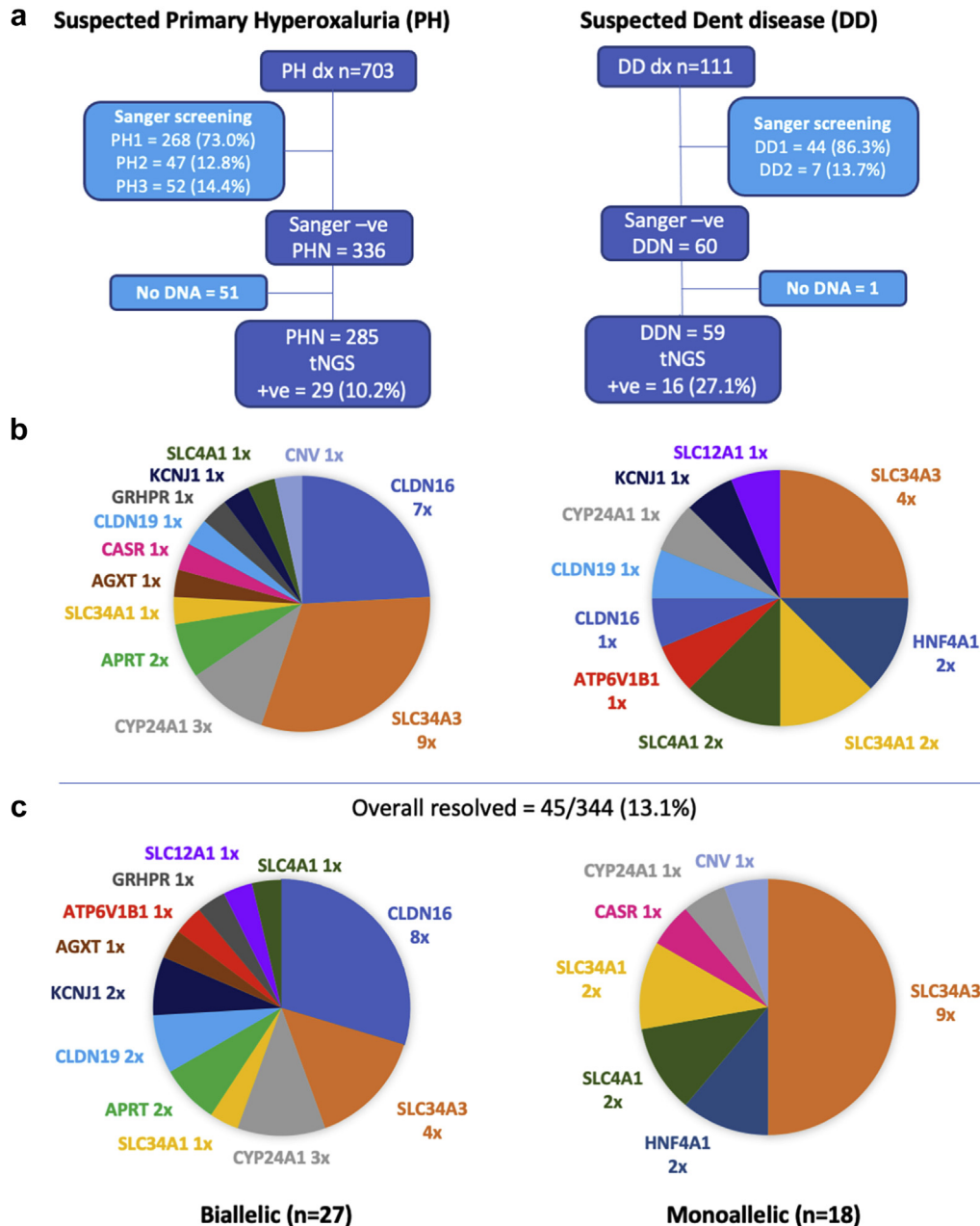


Figure 1. Flow chart showing the design of the study for the suspected primary hyperoxaluria (PH) and Dent disease (DD) populations. (a) The composition of the Sanger-resolved populations and number of PH-negative (PHN) and DD-negative (DDN) patients screened with the targeted next generation sequencing (tNGS) panel are shown. (b) Mutated genes detected from the tNGS of the PHN (left) and DDN (right) populations. (c) An overall summary of the associated genes in the resolved biallelic (left) and monoallelic (right) families.

detected (Figure 2c⁴⁹; Table 2). All the biallelic cases had USD, 3 had NC, but none had ESKD (Figure 3a). The biallelic *SLC34A1* case was from the PHN cohort and homozygous. The *SLC34A1* patient had NC but not known USD.⁵⁹ Nine monoallelic *SLC34A3* cases had consistent phenotypes, including 4 with the previously described missense variant p.Ser192Leu.⁶⁰ Two patients had second *SLC34A3* variants that scored as a variant of uncertain significance (VUS) (Tables 1, 3). Of these, 5 had USD, 4 NC, and 2 had a decline in kidney function. Two patients were

monoallelic for *SLC34A1* pathogenic variants; 1 patient also had a *SLC34A1* VUS, and the second patient had a *SLC34A3* VUS (Tables 1, 3).

Infantile Hypercalcemia Due to 24-Hydroxylase Deficiency

CYP24A1 encodes the enzyme 24-hydroxylase, which metabolizes the active form of vitamin D to an inactive one. Biallelic pathogenic variants have been associated with hypophosphatemia, hypercalcemia, hypercalciuria, NC, and USD,⁴⁰ and monoallelic disease has

Table 1. Clinical and genetic details of likely resolved families

Gene	Pedigree ID	Allele 1 ^a	Allele 2 ^a	Ethnicity ^b (sex)	Age at first stone	No. stones ^c	Stone comp ^d	ESKD (E) or eGFR, age ^e	NC ^f	U/Ca ^g	^h U/Ox	U/pH ⁱ	U/Cit ^j	Comments ^k
Biallelic														
<i>AGXT</i>	PHN244	c.(1_358del) (p.Met1fs)	c.(1_358del) (p.Met1fs)	White (F)	3 yr	3	CaOx	110, 10 yr	N	57	114	7.0	529	Parents confirmed heterozygous carriers
<i>APRT</i>	PHN201	c.3G>C (p.Met1?)	c.3G>C (p.Met1?)	So Asian (F)	NA	Mult	NA	E, 45 yr	Y, 45y	Anuric	Anuric	Anuric	Anuric	
	PHN2-1	c.81-3C>G (p.Asp28?)	c.81-3C>G (p.Asp28?)	White (M)	NA	2	NA	E, 51 yr	Y, 51y	Anuric	Anuric	6.5	Anuric	Very low APRT, blood spot assay
	PHN2-2	c.81-3C>G (p.Asp28?)	c.81-3C>G (p.Asp28?)	White (M)	-	0	-	E, 50 yr	N	Anuric	Anuric	Anuric	Anuric	Crystals on biopsy
<i>ATP6V1B1</i>	DDN55	c.1037C>G (p.Pro346Arg)	c.1037C>G (p.Pro346Arg)	Mid East (F)	2 mo	Mult	CaOx, AP	35, 9 mo	Y, 6m	1081	286	7.5 - 8	NA	Sensorineural deafness, 7 mo
<i>CLDN16</i>	PHN193	c.293G>A (p.Cys98Tyr)	c.293G>A (p.Cys98Tyr)	So Asian (M)	-	0	-	E, 34 yr	Y, 34y	NA	12.5	NA	NA	
	PHN87	c.338G>T (p.Cys113Phe)	c.338G>T (p.Cys113Phe)	So Asian (M)	6 mo	Mult	CaOx	E, 17 yr	Y, 17y	190 mg/g	128	NA	NA	Hypocalcemic tetany, seizures, deafness
	PHN208	c.359G>A (p.Cys120Tyr)	c.359G>A (p.Cys120Tyr)	Hispanic (M)	-	0	-	NA	Y, 6y	5.5	74.3	NA	315	Parapelvic renal cysts
	PHN13	c.427+5G>A (p.Leu143?)	c.427+5G>A (p.Leu143?)	SE Asian (M)	13 yr	Mult	NA	E, 21 yr	N	234 mg/24 h	37 mg/24 hr	NA	NA	
	PHN38	c.445C>T (p.Arg149*)	c.445C>T (p.Arg149*)	Mid East (M)	NA	NA	NA	NA	NA	NA	57	NA	NA	
	PHN223	c.445C>T (p.Arg149*)	c.445C>T (p.Arg149*)	Mid East (F)	16 yr	NA	NA	NA	Y, 16y	NA	NA	NA	NA	
	PHN226	c.571G>A (p.Gly191Arg)	c.571G>A (p.Gly191Arg)	Mid East (F)	1.5 yr	NA	NA	NA	Y, 4y	NA	NA	NA	NA	
	DDN28	c.646C>T (p.Arg216Cys)	c.646C>T (p.Arg216Cys)	So Asian (M)	NA	NA	NA	E, 2 yr	Y, 2y	486 mg/24 h	126	6	NA	<i>SLC4A1</i> : p.Glu906Gln
<i>CLDN19</i>	DDN60	c.392T>G (p.Leu131Arg)	c.392T>G (p.Leu131Arg)	AA (M)	-	0	-	83, 11 yr	Y, 11y	NA	NA	7	NA	Rickets, eye glasses, 11 yr
	PHN112	c.535G>A (p.Gly179Ser)	c.535G>A (p.Gly179Ser)	So Asian (M)	2 yr	Mult	CaOx	E, 16 yr	Y, 16y	NA	3.1 mg/24 h	NA	NA	High myopia
<i>CYP24A1</i>	PHN10	c.364G>T (p.Glu122*)	c.1226T>C (p.Leu409Ser)	White (M)	-	0	-	91, 4 yr	Y, 1y	4.8	89	7	189	
	PHN42	c.428_430del (p.Glu143del)	c.1186C>T (p.Arg396Trp)	White (M)	17 yr	NA	NA	80, 17 yr	Y, 16y	288	83	7	416	Proven biallelic, BRC
	PHN28	c.1226T>C (p.Leu409Ser)	c.1226T>C (p.Leu409Ser)	White (M)	36 yr	1	CaOx	E, 43 yr	Y, 36y	369	40.5	5.7	329	
<i>GRHPR</i>	PH2-6	c.864_865delTG (p.Val289fs20*)	c.214_493del (p.Gly72fs)	Chinese (F)	17 yr	3	CaOx	E, 28 yr	NA	Anuric	Anuric	Anuric	Anuric	
<i>KCNJ1</i>	PHN213	c.562C>A (p.Arg188Ser)	c.562C>A (p.Arg188Ser)	White (F)	NA	NA	NA	173, 11 yr	Y, 11y	445 mg/g	37 mg/g	7.1	898	<i>BSND</i> : p.Gly304Arg
	DDN36	c.1058dupC (p.His354Serfs)	c.788T>G (p.Ile263Ser)	White (M)	57 yr	NA	CaOx	32, 59 yr	Y, 58y	178	40	6.2	261	
<i>SLC12A1</i>	DDN13	c.769G>A (p.Gly257Ser)	c.1424G>A (p.Cys475Tyr)	White (M)	3 yr	NA	NA	83, 3 yr	Y, 3y	6.5	78mg/g	7	212mg/g	
<i>SLC34A1</i>	PHN233	c.1466A>G (p.Tyr489Cys)	c.1466A>G (p.Tyr489Cys)	Icelandic (F)	NA	NA	NA	110, 7 yr	Y, 4y	605 mg/g	356 mg/g	NA	202	MSK
<i>SLC34A3</i>	DDN6	c.413C>T [#] (p.Ser138Phe)	c.448+1G>A (p.Lys149?)	White (F)	17 yr	Mult	COD/COM	38, 19 yr	Y, 17y	233	55	6.4	127	

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Table 1. (Continued) Clinical and genetic details of likely resolved families

Gene	Pedigree ID	Allele 1 ^a	Allele 2 ^a	Ethnicity ^b (sex)	Age at first stone	No. stones ^c	Stone comp ^d	ESKD (E) or eGFR, age ^e	NC ^f	U/Ca ^g	^h U/Ox	U/pH ⁱ	U/Cit ^j	Comments ^k
		c.1576_1578del [#] (p.Leu527del)												
	DDN39	c.560+23_561-42del (p.Arg187?)	c.1058G>T (p.Arg353Leu)	White (F)	16 yr	1	NA	61, 18 yr	Y, 16y	342 mg	NA	NA	NA	
	DDN33	c.1247delT (p.Leu416Profs)	c.1247delT (p.Leu416Profs)	SE Asian (M)	8 yr	Mult	CaOx	103, 11 yr	Y, 8y	15.4	51	5.5-7	257-608	
	DDN41	c.1453C>T (p.Arg485Cys)	c.1454G>A [#] (p.Arg485His)	White (M)	16 yr	~50	COD/COM	101, 37 yr	N	376	NA	NA	NA	
			c.1585A>T [#] (p.Ile529Phe)											
<i>SLC4A1</i>	DDN57	c.2573C>T (p.Ala858Asp)	c.2573C>T (p.Ala858Asp)	Mid East (M)	NA	Mult	AP	92.5, 7 yr	Y, 7y	153 mg/g	NA	8.5	452	
Monoallelic														
<i>CASR</i>	PHN31	c.649G>T (p.Asp217Ty)	ND	African (M)	-	0	-	142, 3 yr	Y, 1y	12	112	7.2	958	HS, <1y, congenital HPT; <i>SLC12A1</i> , p.Gly397Asp
<i>CYP24A1</i>	DDN51	c.469C>T (p.Arg157Trp)	ND	White (F)	19 yr	1	NA	>90, 27 yr	Y, 19y	339	28	6.9	495	BRC, <i>SLC34A1</i> : p.Ala133Val, <i>CYP24A1</i> : p.Arg157Gln
<i>HNF4A</i>	DDN12	c.253C>T (p.Arg85Trp)	ND	White (M)	NA	NA	NA	75, 10 yr	Y, 16y	8.2	59.1	7.0	1893	Fanconi, rickets, glucosuria, UP 30,
	DDN7	c.253C>T (p.Arg85Trp)	ND	White (M)	NA	NA	NA	75, 6 yr	Y, 11y	8.2	60.4	6.6	1168	Fanconi, severe bone disease, UP 100
<i>SLC34A1</i>	DDN61	c.241dupG (p.Glu81Glyfs)	ND	White (F)	-	0	-	98, 3 yr	Y, 15m	NA	NA	NA	NA	<i>SLC34A1</i> : c.1175-3C>A
	DDN26	c.460_480dup (p.Ile154_Val160dup)	ND	Brazil (F)	7 yr	4	NA	125, 15 yr	Y, 7y	4.2	NA	NA	NA	UTI, 7y, RBP slightly high, <i>SLC34A3</i> : c.561-8G>A <i>SLC26A1</i> : c.577-1G>A
<i>SLC34A3</i>	PHN245	c.(1-?)_(1797+)del (p.Met1fs)	ND	White (M)	NA	NA	NA	NA	Y, 6y	3.7	100	8	32	<i>SLC34A3</i> : c.305-7G>A
	PHN32	c.575C>T (p.Ser192Leu)	ND	White (F)	-	0	-	NA	Y, 10y	4.6	100	7.0	558	
	PHN180	c.575C>T (p.Ser192Leu)	ND	White (M)	48 y	3	NA	31, 56 yr	N	118	51	6.1	299	
	PHN239	c.575C>T (p.Ser192Leu)	ND	White (M)	35 y	Mult	NA	42, 62 yr	NA	84	135	5.4	392	<i>SLC34A3</i> : p.Pro571Ser <i>SLC3A1</i> : c.1136+2T>C
	PHN250	c.575C>T (p.Ser192Leu)	ND	NA (M)	7 mo	3	CaOx	NA	NA	NA	NA	NA	NA	
	PHN219	c.846G>A (p.Pro282?)	ND	White (M)	4 yr	2	NA	NA	NA	5.2	79.5	7.0	527	

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Table 1. (Continued) Clinical and genetic details of likely resolved families

Gene	Pedigree ID	Allele 1 ^a	Allele 2 ^a	Ethnicity ^b (sex)	Age at first stone	No. stones ^c	Stone comp ^d	ESKD (E) or eGFR, age ^e	NC ^f	U/Ca ^g	^h U/Ox	U/pH ⁱ	U/Cit ^j	Comments ^k
	PHN274	c.1454G>A [#] (p.Arg485His) c.1585A>T [#] (p.Ile529Phe)	ND	White (F)	<18 yr	Mult.	CaOx	-	Y, 36y	343	120	>8	534	MSK
	PHN156	c.1246_1247del (p.Leu417Thrfs)	ND	White (M)	12 yr	1	CaOx	90, 12 yr	Y, 12y	9.2	68	7.0	488	Autism
	PHN53	c.1623G>A (p.Trp541*)	ND	White (F)	-	0	-	117, 30 mo	Y, 1y	8.9	74	7.0	523	Failure to thrive, 9 mo; <i>CYP24A1</i> , p.Glu143del
<i>SLC4A1</i>	PHN152	c.1765C>T (p.Arg589Cys)	ND	White (M)	-	0	-	150, 6 yr	Y, 6y	4.3	100	7.0	<73	Urinary incontinence
	DDN8	c.2726T>C (p.Met909Thr)	ND	White (M)	-	0	-	81, 6 yr	Y, 5y	3.1	48.9	7.4	<48	Hematuria, prenatal hydronephrosis
Chr4q del	PHN20	chr4 (85,553,401-104,356,614) 18.8MB	ND	White (M)	6 mo	Mult	NA	139, 6 mo	N	466	399 mg/g	6	NA	Failure to thrive

Biochemical values outside the normal range are shown in boldface type. NA, information not available.

^aAllele: # = variants suspected of being on the same allele; ND, not detected.

^bEthnicity (sex): Mid, middle; So, south; SE, south east; AA, African American; (F), female; (M), male; NA, information not available.

^cNo. stones, total number of stones observed; Multi, multiple.

^dStone comp, stone composition; CaOx, calcium oxalate; AP, apatite; COD/COM, calcium oxalate dihydrate/calcium oxalate monohydrate.

^eESKD, eGFR, age: E, end-stage kidney disease with age indicated; yr, year; mo, month; estimated glomerular filtration rate (eGFR), value and age indicated; eGFR calculated with Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (ml/min per 1.73 m²) or Full Age Spectrum (FAS) pediatric equation for patients <1 yr.

^fNC, nephrocalcinosis; Y, yes and age first detected; y, year; m, month; N, no.

^gU/Ca, urine calcium, shown as mg/24 h when ≥18 yr or as mg/kg per 24 h when <18 yr (underlined), unless otherwise shown.

^hU/Ox, urine oxalate, shown as mg/24 h when ≥18 yr or as mg/1.73 m² when <18 yr (underlined), unless otherwise shown.

ⁱU/pH, urine pH

^jU/Cit, urine citrate shown in mg/24 h when >18 yr or as mg/g creatinine when <18 yr (underlined). Creatinine normalization (mg/g creatinine).

^kComments: BRC, bilateral renal cysts; RBP, retinol binding protein; HPT, hyperparathyroidism; HS, hypocalcemic seizures; MSK, medullary sponge kidney; UTI, urinary tract infection; UP, urinary protein. Variants that may be significant to the phenotype are shown in boldface type.

Table 2. Details of the described pathogenic variants

Gene ^a	Disease ^b	Family ID	Zygoty ^c	Variant description	Variant type ^d	Pub ^e	GnomAD frequency ^f	Splicing evaluation ^g		Missense evaluation ^h			ACMG evaluation ⁱ	
								HSF	BDGP	Pred	Ortho	Dom	Class	Evidence
<i>AGXT</i>	PH1	PHN244	Hom	c.1_358del (p.Met1fs)	L Del	N	0	NA	NA	NA	NA	NA	Path Ib	PVS1, PM2, PM3, PP4
<i>APRT</i>	APRTd	PHN201	Hom	c.3G>C (p.0?)	NonStart	N	2/146792	NA	NA				Path Ib	PVS1, PM2, PM3
		PHN2	Hom	c.81-3C>G (p.Asp28fs)	Splice	N	3/197204	87.65 to 77.36	0.23 to <0.1	NA	NA	NA	LP IV	PM2, PM3, PP1-M, PP3, PP4
<i>ATP6V1B1</i>	dRTA	DDN55	Hom	c.1037C>G (p.Pro346Arg)	Mis	36	4/250624	NA	NA	6/6	7/7	NA	Path II	PS1, PS3, PM2, PM3, PP4, PS4
<i>CASR</i>	HHC1	PHN31	Het [~]	c.649G>T (p.Asp217Tyr)	Mis	ClinVar (x2 LP)	0	NA	NA	6/6	6/7	NA	LP VI	PM2, PP2, PP3, PP4, PP5
<i>CLDN16</i>	FHHNC	PHN193	Hom	c.293G>A (p.Cys98Tyr)	Mis	N	0	NA	NA	5/6	8/8	NA	LP V	PM2, PM3, PP2, PP3, PP4
		PHN87	Hom	c.338G>T (p.Cys113Phe)	Mis	N	0	NA	NA	4/6	6/6	NA	LP V	PM2, PM3, PP2, PP3, PP4
		PHN208	Hom	c.359G>A (p.Cys120Tyr)	Mis	N	8/251470	NA	NA	6/6	6/6	7/7	LP	PM1, PM3, PP2, PP3, PP4
		PHN13	Hom	c.427+5G>A (p.Leu143?)	Splice	37	4/251366	76.03 to 49.49	0.88 to 0.05	NA	NA	NA	Path IIIb	PS1, PM2, PM3, PP3, PP4
		PHN38, PHN223	Hom	c.445C>T (p.Arg149*)	Nons	38	1/251490	NA	NA	NA	NA	NA	Path Ia	PVS1, PS1, PM2, PM3, PP4
		PHN226	Hom	c.571G>A (p.Gly191Arg)	Mis	38	NA	NA	0.92 to 0.92	6/6	8/8	NA	Path II	PS1, PS4, PM3, PP2, PP3, PP4
		DDN28	Hom	c.646C>T (p.Arg216Cys)	Mis	39	3/282812	NA	NA	6/6	8/8	NA	Path IIIb	PS1, PM2, PM3, PP2, PP3, PP4
<i>CLDN19</i>	FHHNC	DDN60	Hom	c.392T>G (p.Leu131Arg)	Mis	N	0	NA	NA	6/6	6/7	6/7	LP IV	PM1, PM2, PM3, PP2, PP3, PP4
		PHN112	Hom	c.535G>A (p.Gly179Ser)	Mis	13	3/206108	NA	NA	6/6	7/7	7/7	Path IIIa	PS1, PM1, PM2, PM3, PP3, PP4
<i>CYP24A1</i>	HCINF1	PHN10	C Het	c.364G>T (p.Glu122*)	Nons	N	1/250584	NA	NA	NA	NA	NA	Path Ic	PVS1, PM2, PP4
		PHN42, PHN53, PHN200	C Het, Het [~] , Het	c.428_430del (p.Glu143del)	I/F Del	40	146/282660 (1 hom)	NA	NA	NA	7/7	1/7	Path II ^R	PS1, PS3, PM4, PP4
		DDN51	Het	c.469C>T (p.Arg157Trp)	Mis	41	525/282662	NA	NA	3/6	7/7	1/7	LP II	PS1, PM3, PP4
		PHN42, PHN63, PHN234	C Het, 2x Het	c.1186C>T (p.Arg396Trp)	Mis	40	199/282630 (1 hom)	NA	NA	6/6	7/7	6/7	Path II ^R	PS1, PS3, PP3, PP4
		PHN10, PHN28, PHN68, PHN115	C Het, Hom, 2x Het	c.1226T>C (p.Leu409Ser)	Mis	40	209/282476	NA	NA	6/6	7/7	1/7	Path II ^R	PS1, PS3, PM3, PP3, PP4
<i>GRHPR</i>	PH2	PH2-6	C. Het	c.214_493del (p.Gly72fs)	L Del	N	0	NA	NA	NA	NA	NA	Path Ic	PVS1, PM2, PP4
		PH2-6	C. Het	c.864_865delTG (p.Val289fs20*)	F/S Del	42	11/282828	NA	NA	NA	NA	NA	Path Ia	PVS1, PS1, PP4

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Table 2. (Continued) Details of the described pathogenic variants

Gene ^a	Disease ^b	Family ID	Zygoty ^c	Variant description	Variant type ^d	Pub ^e	GnomAD frequency ^f	Splicing evaluation ^g		Missense evaluation ^h			ACMG evaluation ⁱ	
								HSF	BDGP	Pred	Ortho	Dom	Class	Evidence
<i>HNF4A</i>	FRTS4	DDN12, DDN7	2x Het	c.253C>T (p.Arg85Trp)	Mis	43	0	NA	NA	6/6	7/7	9/9	Path II	PS1, PS4, PM1, PP4
<i>KCNJ1</i>	BARTS2	PHN213	Hom	c.562C>A (p.Arg188Ser)	Mis	N	1/249916	NA	NA	6/6	6/6	9/10	LP V	PM2, PM3, PP3, PP4
		DDN36	C Het	c.788T>G (p.Ile263Ser)	Mis	N	0	NA	NA	6/6	6/6	6/10	LP V	PM1, PM2, PP2, PP3, PP4
		DDN36	C Het	c.1058dupC (p.His354Serfs)	F/S Dup	44	14/282766	NA	NA	NA	NA	NA	Path Ia	PVS1, PS1, PP4
<i>SLC12A1</i>	BARTS1	DDN13	C Het	c.769G>A (p.Gly257Ser)	Mis	45	1/31402	NA	NA	5/6	6/6	7/7	LP II	PS1, PM2, PP3, PP4
		DDN13	C Het	c.1424G>A (p.Cys475Tyr)	Mis	13	0	NA	NA	5/6	6/6	NA	LP II	PS1, PM2, PP3, PP4
<i>SLC34A1</i>	HCINF2	DDN61	Het	c.241dupG (p.Glu81Glyfs)	F/S Dup	N	1/248792	NA	NA	NA	NA	NA	Path Ic	PVS1, PM2, PP4
		DDN26	Het	c.460_480dup (p.Ile154_Val160dup)	I/F Del	46	5/251404	NA	NA	NA	NA	NA	LP II	PS1, PM2, PM4
		PHN233	Hom	c.1466A>G (p.Tyr489Cys)	Mis	8	1/250692	NA	NA	6/6	7/7	5/8	LP II	PS4, PM3, PP3, PP4
<i>SLC34A3</i>	HHRH	PHN245	Het	c.(1-?)_(1797+) del(p.Met1fs)	L del	N	0	NA	NA	NA	NA	NA	Path Ib	PVS1, PM2, PM3, PP4
		DDN6	C Het*	c.413C>T (p.Ser138Phe)	Mis	47	30/273572	NA	NA	5/6	6/6	5/8	LP II	PS1, PM3, PP3, PP4
		DDN6	C Het*	c.448+1G>A (p.Lys149?)	Mis	48	41/266562	72.6 to 45.4	0.1 to 0	NA	NA	NA	Path Ia	PVS1, PS1, PM3, PP4
		DDN39	C Het	c.560+23_561-42del (p.Arg187?)	Splice	49	50/240582	NA	NA	NA	NA	NA	LP III	PS1, PP3, PP4
		PHN32, PHN180, PHN239, PHN250	4x Het	c.575C>T (p.Ser192Leu)	Mis	50	99/214524	NA	NA	6/6	7/7	1/8	Path II	PS1, PS3, PS4, PP4
		PHN219	Het	c.846G>A (p.Pro282?)	Splice	50	7/280346	88.39 to 78.31	0.78 to 0.11	NA	NA	NA	LP III	PS1, PP3, PP4
		DDN39	C Het	c.1058G>T (p.Arg353Leu)	Mis	50	4/243200	NA	NA	4/6	6/6	NA	LP II	PS1, PM2, PP4
		PHN156	Het	c.1246_1247del (p.Leu417Thrfs)	F/S Del	ClinVar 1x LP	14/248800	NA	NA	NA	NA	NA	Path Id	PVS1, PP4, PP5
		DDN33	Hom	c.1247delT (p.Leu416Profs)	F/S Del	N	1/248562	NA	NA	NA	NA	NA	Path Ib	PVS1, PM2, PM3, PP4
		DDN41	C Het*	c.1453C>T (p.Arg485Cys)	Mis	N	151/277496	NA	NA	6/6	6/7	5/8	LP V	PM3, PM5, PP3, PP4
<i>SLC34A3</i>	HHRH	DDN41, PHN274	C Het*, Het*	c.1454G>A (p.Arg485His)	Mis	12	769/277194 (3 hom)	NA	NA	6/6	6/7	5/8	LP II	PS1, PM3, PP4
		DDN6	C Het*	c.1576_1578del (p.Leu527del)	I/F Del	47	43/253996	NA	NA	NA	6/6	5/8	Path Ib	PS1, PM3, PM4, PP3, PP4
		DDN41, PHN274	C Het*, Het*	c.1585A>T (p.Ile529Phe)	Mis	12	668/243972 (2 hom)	NA	NA	1/6	4/7	NA	LP II	PS1, PM3, PP4

(Continued on following page)

Table 2. (Continued) Details of the described pathogenic variants

Gene ^a	Disease ^b	Family ID	Zygoty ^c	Variant description	Variant type ^d	Pub ^e	GnomAD frequency ^f	Splicing evaluation ^g		Missense evaluation ^h			ACMG evaluation ⁱ	
								HSF	BDGP	Pred	Ortho	Dom	Class	Evidence
SLC4A1	dRTA	PHN53	Het [^]	c.1623G>A (p.Trp541*)	Nons	N	1/158290	NA	NA	NA	NA	NA	Path lc	PVS1, PM2, PP4
		PHN152	Het	c.1765C>T (p.Arg589Cys)	Mis	51	0	NA	NA	6/6	7/7	8/12	LP II	PS1, PM1, PM2, PP4
		DDN57	Hom	c.2573C>T (p.Ala858Asp)	Mis	52	18/250988	NA	NA	5/6	5/7	NA	Path II	PS1, PS3, PM3, PP4
		DDN8	Het	c.2726T>C (p.Met909Thr)	Mis	53	0	NA	NA	5/6	7/7	4/6	Path II	PS1, PS3, PM2, PP4
Chr4q del	NA	PHN20	Het	chr4 (85,553,401-104,356,614) 18.8MB	L Del	N	N	NA	NA	NA	NA	NA	LP I	PSV1, PM2

NA, not applicable.

^aGene: nucleotide and protein Accession Numbers are shown in Table S3.

^bDisease: Online Mendelian Inheritance in Man (OMIM) terms used. PH, primary hyperoxaluria; APRTd, adenine phosphoribosyltransferase deficiency; dRTA, distal renal tubular acidosis; HHC1, hypocalciuric hypercalcemia; familial, type I, FHHNC, familial hypomagnesemia with hypercalciuria and nephrocalcinosis; HCINF, infantile hypercalcemia; FRTS, Fanconi renotubular syndrome; BARTS, Bartter syndrome; HRRH, hereditary hypophosphatemic rickets with hypercalciuria.

^cZygoty: Hom, homozygous; Het, heterozygous; C Het, compound heterozygous. ^Complex genotype; *3 alleles detected.

^dVariant type: L del, large deletion; NonStart, start codon substitution; Mis, missense; Nons, nonsense; I/F Del, inframe deletion; F/S Del, frameshifting deletion; F/S Dup, frameshifting duplication.

^ePub: prior description in a publication; N, novel variant, description in ClinVar if unpublished; LP, likely pathogenic.

^fGnomAD frequency: frequency in the gnomAD database of "normal individuals"; hom, homozygous descriptions.

^gSplicing evaluation: HSF, Human Splice Finder; BDGP, Berkley Drosophila Gene Project, for both normal and variant score shown, and where appropriate, N is the score of novel site generated, NA, not applicable.

^hMissense evaluation: Pred, fraction of predicted damaging pathogenicity scores from the following: SIFT, PolyPhen-2 HVAR, MutationTaster, Mutation Assessor, FATHMM, and FATHMM MKL. Ortho, fraction matching the human sequence in a multisequence alignment (MSA) of orthologs from mammals to fish. Dom, fraction matching the human sequence MSA of conserved domains, NCBI database, NA, not applicable.

ⁱACMG evaluation: Class, pathogenic classification based on the American College of Medical Genetics (ACMG) guidelines for interpretation of sequence variants: Path, pathogenic; LP, likely pathogenic, with subclasses shown. Evidence, ACMG evidence supporting the interpretation of sequence variant classification. The evidence is classed as follows: PVS1, pathogenic very strong; PS, pathogenic strong; PM, pathogenic moderate; PP, pathogenic supportive (see Richards *et al.*¹⁹ for details).

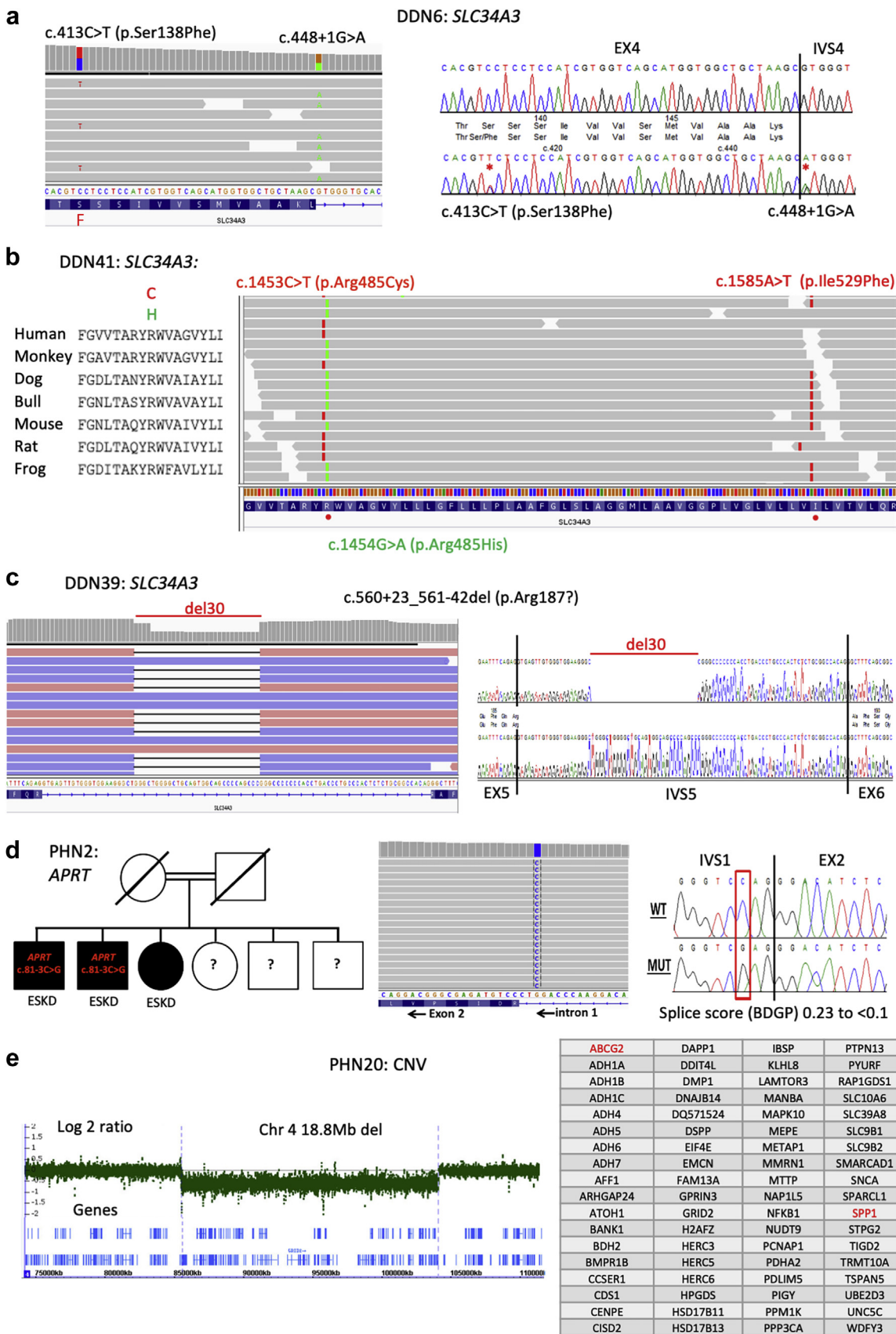


Figure 2. Examples of genetic results from 5 families. (a) DDN has 3 *SLC34A3* variants: c.413C>T (p.Ser138Phe); c.1576_1578del (p.Leu527del); and c.448+1G>A (p.Lys149?). Analysis of data from other families (not shown) and published data⁴⁷ indicated that c.413C>T (p.Ser138Phe) and c.1576_1578del (p.Leu527del) are likely on the same allele. Analysis of the tNGS reads showed that c.413C>T (p.Ser138Phe) (continued)

occasionally been described.⁷⁸ Biallelic *CYP24A1* pathogenic changes were identified in 3 subjects (1 of whom was homozygous) with 1 novel variant (Tables 1, 2). All had NC, 2 were children or young adults, but only 1 experienced ESKD. One patient with a typical *CYP24A1*-deficiency phenotype was monoallelic (Table 1; Figure 3C).

Adenine Phosphoribosyltransferase Deficiency (APRTd)

Biallelic *APRT* pathogenic variants result in accumulation of the insoluble purine 2,8-dihydroxyadenine (DHA) in the kidney, USD, and CKD.^{79–81} Two families were homozygous for novel *APRT* pathogenic variants, a start codon substitution (PHN201), or an atypical splicing variant (PHN2) (Tables 1, 2). PHN2 is a consanguineous family that includes 3 siblings with ESRD; the *APRT* c.81-3C>G atypical splicing change was predicted to greatly weaken the donor site (Figure 2d). Subsequent biochemical analysis confirmed very low APRT levels.

Barter Syndrome

Biallelic pathogenic variants to 6 genes cause Bartter syndrome, a disease characterized by impaired sodium reabsorption in the thick ascending loop of Henle that results in salt wasting, hypokalemic metabolic alkalosis, and hypercalciuria. Three families (2 DDN) had biallelic pathogenic variants to Bartter disease genes, 2 to *KCNJ1* encoding the ROMK channel⁶⁵ (1 homozygous and 2 novel missense changes) and 1 to *SLC12A1* encoding NKCC2.⁸³ Two of the subjects were children, all had NC, and 2 had USD (Figure 3b).

Distal Renal Tubular Acidosis (dRTA)

Biallelic variants were found in 2 dRTA genes, *ATP6V1B1*³⁶ and *SLC4A1*.⁸⁴ Each of these DDN cases was homozygous for known pathogenic variants. In addition, 2 children with NC had single known *SLC4A1* pathogenic variants⁵¹ (Tables 1, 2).

Dominant Fanconi Syndrome

Two patients with Fanconi syndrome, severe bone disease, and NC had a single known pathogenic variant to the transcription factor *HNF4A*⁸⁵ (Tables 1, 2).

Autosomal Dominant Hypocalcemia

One subject with hypocalcemic seizures and NC as an infant had a single known pathogenic variant to the calcium-sensing receptor gene, *CASR* (Tables 1, 2).⁸⁶

Copy Number Variant

One infant with multiple stones and failure to thrive was found to have an 18.8-Mb chromosome 4 deletion containing 72 genes. This was initially detected by tNGS, due to CNV of *ABCG2* and *SPP1*, and was confirmed by microarray analysis (Figure 2e).

Other USD/NC Gene Variants

As well as the likely solved cases, variants of interest were detected in a further 42 families, including 11 with more than 1 variant (Tables 3^{61–77}, 4). In addition, 9 likely solved families had additional variants of interest (Table 1). Variants that may be significant to the phenotype are shown in boldface type in Tables 1 to 3. These variants of interest included 29 previously described pathogenic changes or truncating variants to known USD genes. As some examples, the phenotype of 3 patients with rare monoallelic *SLC34A1* variants (1 truncating) and 3 with a single *SLC34A3* VUS were not believed to be completely explained by these variants (Tables 3, 4; Figure S1A). Another 10 subjects were monoallelic for known (7) or suspected (3) pathogenic *CYP24A1* variants (3), including 4 with other variants of interest (Table 4; see also Discussion). The significance of an *HNF4A* missense change predicted to alter splicing (Table 3) and described as a VUS in ClinVar was unclear in PHN71. Single pathogenic variants to cystinuria genes have been described to sometimes act dominantly⁸⁷ and were detected in 4 families, but none had a documented history of cystine stones.

Figure 2. (continued) and c.448+1G>A (p.Lys149?) are on different alleles (left), with the Sanger sequence shown (right), and so this patient has a biallelic genotype. (b) Patient DDN41 also has 3 *SLC34A3* variants: c.1453C>T (p.Arg485Cys); c.1454G>A (p.Arg485His); and c.1585A>T (p.Ile529Phe). The conservation of p.Arg485 is shown in multisequence alignment (left), with the phase data from the targeted next generation sequencing (tNGS) reads showing that c.1453C>T (p.Arg485Cys) and c.1585A>T (p.Ile529Phe) are on the same allele and c.1454G>A (p.Arg485His) is on the other allele. (c) Patient DDN39 has 2 *SLC34A3* variants, an intronic deletion of 30 bp within IVS5, c.560+23_561-42del (p.Arg187?), plus the missense variant c.1058G>T (p.Arg353Leu). The deletion shown in next generation sequencing (NGS) reads (left) and Sanger sequence (right) leaves a very small intron (65 bp) that may not be excised efficiently.⁴⁹ (d) In pedigree PHN2 (left), 3 siblings have end-stage kidney disease (ESKD), and in 2 (where samples were available; PHN2-1 and PHN2-2) the atypical splicing variant c.81-3C>G (p.Asp28?) to *APRT* was detected in homozygosity, shown by NGS (center) and Sanger sequence (right). This novel variant in IVS1 is predicted to eliminate the splice acceptor site. (e) In PHN20 a CNV deletion was detected with the genes *ABCG2* and *SPP1* (chr 4q) using the 90-gene panel. Follow-up microarray analysis detected a 18Mb deletion (left) containing 72 genes (right).

DISCUSSION

In this cohort of patients clinically suspected of PH or DD but lacking pathogenic variants in their respective causative genes, a tNGS panel determined that 10.2% (PHN) or 27.1% (DDN) were instead due to pathogenic variants in other known USD/NC-associated genes. Primary hyperoxaluria and Dent disease have quite different pathogenic origins, as do the other diseases that we identified in this cohort. However, USD, NC, and CKD are often present, with increased urinary calcium excretion a common feature.⁷ Our study demonstrates the value of using a broad approach for genetic screening of patients suspected of monogenic USD, as the likely genetic cause is often not easy to discern from clinical or biochemical data, which can be subject to biologic variability and can be difficult to interpret, especially in younger children. Thus, our study supports the increased use of clinical molecular testing in suspected monogenic USD patients.^{11–13,58,88,89} Clinical genetic testing now costs only a few hundred dollars in the United States and most often is covered by insurance. As well as the positive results of a firm diagnosis, there can be cost savings from decreasing the need for unnecessary follow-up radiologic and/or biochemical screening, and use of ineffective treatments that may have significant side effects.⁸⁹ However, for commercial testing, interpretation in a patient-specific context is an important consideration. Ultimately, the ordering provider must have expertise or access to expertise to interpret results in a patient specific manner.⁸⁹

Of our total 67 suspected DD families now resolved (Sanger and NGS), 16 (23.9%) are due to defects to other genes, which is twice as many as for DD2 (7; 10.4%), with 4 (6.0%) cases accounted for by *SLC34A3* pathogenic variants. Among the suspected PH families, 29 of 396 (7.3%) were due to other genes, with *SLC34A3* and *CLDN16* the most commonly involved (9 [2.3%] and 7 [1.8%] cases, respectively). Pathogenic variants to *CLDN16*, *CLDN19*, *SLC34A3*, and *KCNJ1* were found to account for both PH- and DD-suspected patients. Because patients were initially recruited with somewhat liberal criteria in order to maximize sensitivity, the newly resolved families add to our knowledge of the groups of monogenic USD/NC that can present with similar clinical features.

Increased plasma oxalate or urinary oxalate excretion are reliable indicators of PH. However, plasma oxalate can be challenging to measure, because it is offered only by relatively few reference laboratories and requires special handling after blood draw and during shipment. In addition, interpretation of plasma or urine oxalate values can be challenging in young

infants, and evaluation of PH patients who present after kidney failure requires access to a reliable plasma oxalate assay. Although LMWP is characteristic of DD, specific measurement of low-molecular-weight proteins is often complicated because it is a referral test at many centers. In addition, moderate LMWP is commonly present in other causes of NC that involve the proximal nephron, making differentiation from DD even more challenging if only routine urinary total protein and albumin measurements are available. Therefore, although initial suspicion of PH and DD were attributed to elevated urinary excretion or LMWP, respectively, our study reveals that these markers can be misleading, illustrating the value of genetic testing.

Clarifying the diagnosis led to changes in management. For example, in patients confirmed with PH, definitive treatment with siRNA inhibition of the glycolate oxidase gene, *HAOI* is currently available for PH1 and siRNA inhibition of the lactate dehydrogenase A gene, *LDHA*, is in clinical trials for treatment of PH2 and PH3.^{14,16} For CYP24A1 deficiency, early restriction of dietary calcium and vitamin D are effective in managing hypercalcemia,⁹⁰ and agents are under investigation that can enhance 24-hydroxylase activity. For patients having APRTd, oral administration of allopurinol or febuxostat is highly effective in reducing stones and preserving kidney function. Patients with 2 pathogenic changes to *SLC34A3* provide another example how a correct genetic diagnosis changed management, as the primary treatment for this disorder is phosphorus supplementation, which would not have been considered without this diagnosis. Thus, a definitive molecular diagnosis permits specific and effective treatment interventions. Furthermore, with rapid advances in molecular treatments for rare diseases, a definitive diagnosis facilitates enrollment in clinical trials and early treatment as evolving therapies become available.

Our tNGS gene panel approach, as opposed to broader WES, allowed greater pooling of samples during capture and sequencing, thus reducing the cost. Also, the greater read depth made CNV more readily detectable than via WES. We note that diagnostic protocols using WES usually limit analytic screening to a group of known genes fitting the phenotype (similar to tNGS),¹¹ although the WES approach allows follow-up analysis of the whole exome if subsequently desired.

The use of a comprehensive screening approach to identify all possible pathogenic variants, including novel missense changes and atypical splicing events, is necessary for a rigorous genetic screen. The analysis in this study made full use of normal and disease population databases, variant and splicing

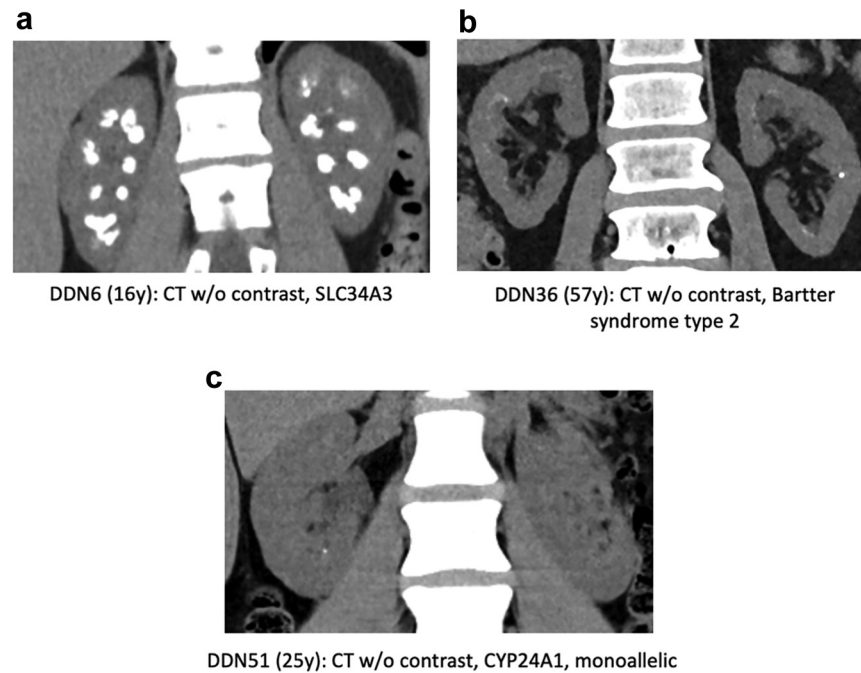


Figure 3. Renal imaging of primary hyperoxaluria–negative (PHN) and Dent disease–negative (DDN) cohort depicting the spectrum of renal phenotypes. (a) Abdominal computed tomography (CT) without (w/o) contrast of DDN6 with biallelic *SLC34A3* pathogenic variants causing HRRH showing diffuse severe medullary nephrocalcinosis (NC). (b) CT of DDN36 with Bartter syndrome type 2 due to *KCNJ1* pathogenic variants showing mild NC plus stones. (c) CT of DDN51 with a monoallelic *CYP24A1* pathogenic variant showing tiny calyceal tip stones.

evaluation tools, and CNV analysis. Results were finally scored according to the ACMG guidelines to determine the significance of detected variants (see [Supplementary Methods](#) for details). Family analysis (possible in a minority of cases) and analysis of single NGS reads, when variants were close together, were helpful for determining the phase of variants ([Figure 2a, b](#)), which is key to determining the pathogenicity of compound heterozygous cases. The advantage of having clinical data in combination with the genetic information was also illustrated by our study, because this enhanced the ability to determine the likely pathogenic significance of variants and whether they fit the phenotype—analysis that is not always possible in a commercial clinical testing setting. In addition, the analysis allowed all rare variants to be considered, including some that may modify the phenotype. However, because the significance of these variants is largely unknown (VUS), we have grouped them separately ([Tables 3, 4](#)). Nevertheless, some of these variants are strong candidates for follow-up research studies in larger cohorts, including their potential role as disease modifiers.

This study differs from earlier ones that reported the results of screening for monogenic USD/NC, because a larger pool of normal population data (gnomAD⁹¹) and collections of information on gene variants in the disease setting (ClinVar) are now available, enhancing the

ability to determine the pathogenic significance of variants. *SLC34A1*: p.Ala133Val was previously described as monoallelically pathogenic, with some *in vitro* data supporting this pathogenic role.^{58,73,88} However, the frequency of this variant in normal populations ([Table 4](#)) and the finding of other significant variants led us to doubt a pathogenic role, although it may be a disease modifier (DDN51, PHN133). In the case of *SLC34A1*: p.Val91_Ala97del, some functional data in the literature and its association with NC in homozygosity suggest a pathogenic role.^{82,92} However, although the frequency is very high in the normal population (1.7%), we found this variant only in a heterozygous state and with no clearly related phenotype ([Tables 3, 4](#)).

Analysis of *SLC34A3* and *SLC34A1* subjects was illustrative of the complexity of monogenic disease. For *SLC34A3*, similar pathogenic variants were found in biallelic and monoallelic cases ([Table 1](#)),^{56–58} and there appeared to be phenotypic overlap. From the available clinical information, the phenotype of the monoallelic *SLC34A3* cases was consistent with this genetic change being a contributing factor; we also note that the monoallelic p.Ser192Leu phenotype is particularly variable,⁶⁰ and some cases had other VUS of interest ([Table 1](#)). However, because of lost to follow up between the initial targeted genetic screening and the current tNGS panel analysis, we were not able to obtain definitive evidence for a renal phosphate handling defect such as low serum phosphorus or increased fractional excretion of

Table 3. Details of other variants of interest

Gene ^a	Family ID ^b	Zygoty ^c	Variant description	Variant type ^d	Pub ^e	GnomAD frequency ^f	Splicing evaluation ^g		Missense evaluation ^h			ACMG evaluation ⁱ	
							HSF	BDGP	Pred	Ortho	Dom	Class	Evidence
<i>ALPL</i>	PHN280	Het	c.1001G>A (p.Gly334Asp)	Mis	61	0	NA	NA	6/6	8/8	6/12	LP II ^R	PS1, PS3, PM2
<i>ALPL</i>	PHN23	Het	c.1069C>T (p.Arg357Trp)	Mis	N	5/251484	NA	NA	4/6	4/8	2/8	VUS	PM2
<i>APRT</i>	DDN5	Het [^]	c.541T>C (p.*181Argexf?*)	NonStop	62	1/250662	NA	NA	NA	NA	NA	LP II ^R	PS1, PM2, PM4
<i>ATP6VOA4</i>	DDN64	Het	c.334C>G (p.Gln112Glu)	Mis	N	10/251446	NA	NA	3/6	6/7	NA	VUS	PP4
<i>ATP6V1B1</i>	PHN54	Het [^]	c.181C>T (p.Gln61*)	Nons	ClinVar 1xP, 1xVUS	2/250526	NA	NA	NA	NA	NA	LP II ^R	PVS1, PM2
	PHN99	Het [^]	c.1155dupC (p.Ile386Hisfs)	F/S Dup	36	1/31018	NA	NA	NA	NA	NA	Path Ia ^R	PVS1, PS1, PM2
<i>BSND</i>	PHN255	Het	c.673C>T (p.Gln225*)	Nons	N	11/282828	NA	NA	NA	NA	NA	VUS	PVS1
	DDN48	Het [^]	c.770A>G (p.Gln257Arg)	Mis	ClinVar 1xVUS	31/282584	NA	NA	1/6	5/7	NA	VUS	
	PHN90	Het	c.859G>T (p.Glu287*)	Nons	ClinVar 2xVUS	1/251428	NA	NA	NA	NA	NA	VUS	PM2, PM4
	PHN213	Het [^]	c.910G>A (p.Gly304Arg)	Mis	N	5/251146	NA	NA	5/6	6/8	NA	VUS	PM2
<i>CLCNKB</i>	PHN212	Het	c.782-2A>G (p.Glu261?)	Splice	63	3/263238	86.3 to 58.4	0.8 to <0.1	NA	NA	NA	Path Ia ^R	PVS1, PS1, PM2, PP3
<i>CYP24A1</i>	PHN144, DN51, PHN237	Hom, C Het, Het [^]	c.470G>A (p.Arg157Gln)	Mis	64	831/ 282662 (1 hom)	NA	NA	3/6	7/7	1/7	VUS	PS3, PM3, PM5, PP4, BS2
	PHN80	Het [^]	c.964G>A (p.Glu322Lys)	Mis	40	11/282854	NA	NA	5/6	7/7	3/7	Path II ^R	PS1, PS3, PP3
	DDN48	Het [^]	c.1339dupA (p.Ile447Asnfs)	F/S Dup	N	3/251438	NA	NA	NA	NA	NA	Path I ^R	PVS1, PM2
	PHN120	Het	c.1385G>A (p.Cys462Tyr)	Mis	N	13/282854 (1 hom)	NA	NA	6/6	6/7	7/7	VUS	PP2, PP3
<i>CYP27B1</i>	PHN157	Het [^]	c.1378delC (p.Leu460Trpfs)	F/S Del	N	N	NA	NA	NA	NA	NA	LP I ^R	PVS1, PM2
<i>HNF4A</i>	PHN71	Het	c.427A>G (p.Ser143Gly)	Mis	ClinVar 3x VUS	14/251066	N 1.99 to 6.23	N 0.06 to 0.73	4/6	7/7	NA	VUS	PP3
	<i>PHN157</i>	Het [^]	c.724G>A (p.Val242Met)	Mis	65	2/249892	NA	NA	4/6	6/7	2/9	VUS	PS1
<i>KCNJ1</i>	<i>DDN46</i>	Het	c.932G>A (p.Arg311Gln)	Mis	66	3/282548	NA	NA	6/6	7/7	10/10	LP II ^R	PS1, PM2, PP3
<i>SLC12A1</i>	PHN31	Het [^]	c.1190G>A (p.Gly397Asp)	Mis	N	0	NA	NA	6/6	6/6	7/7	VUS	PM2, PM5, PP3
<i>SLC12A3</i>	PHN133	Het [^]	c.363G>C (p.Glu121Asp)	Mis	67	257/ 281630 (1 hom)	NA	NA	2/6	5/7	4/7	Path II ^R	PS1, PS3,
	PHN249	Het	c.1963C>T (Arg665Cys)	Mis	68	7/250982	NA	NA	6/6	7/7	7/8	LP II ^R	PS1, PM2, PM3, PP3
<i>SLC22A12</i>	<i>DDN50</i> , <i>PHN77</i>	Het	c.1301G>A (p.Arg434His)	Mis	69	512/ 266952 (1 hom)	NA	NA	5/6	4/6	NA	VUS	PS1, PS3, BS1
<i>SLC26A1</i>	PHN228	Het	c.528C>A (p.Tyr176*)	Nons	N	53/256644	NA	NA	NA	NA	NA	VUS	PVS1
	DDN26	Het [^]	c.577-1G>A (p.Val193?)	Splice	N	2/210338	86.1 to 58.2	0.88 to <0.1	NA	NA	NA	LP II ^R	PVS1, PM2
<i>SLC4A1</i>	PHN280	Het [^]	c.706T>G (p.Phe236Val)	Mis	N	30/279230	NA	NA	5/6	8/8	11/12	VUS	PP3
<i>SLC3A1</i>	<i>PHN99</i> , <i>PHN136</i>	Het [^] , Het	c.1400T>C (p.Met467Thr)	Mis	70	682/ 282552 (4 hom)	NA	NA	5/6	7/7	6/11	Path I ^R	PS1, PS3, PS4
	PHN237	Het [^]	c.161delC (p.Gln55Argfs)	F/S Del	71	17/282536	NA	NA	NA	NA	NA	Path Ia ^R	PVS1, PS1
	PHN239	Het [^]	c.1136+2T>C (p.Arg379?)	Splice	72	24/282546	NA	NA	NA	NA	NA	Path Ia ^R	PVS1, PS1
<i>SLC34A1</i>	PHN88	Het	c.115C>T (p.His39Tyr)	Mis	N	1/248542	NA	NA	4/6	6/7	NA	VUS	PM2
	DDN21, PHN179, PHN222	Het	c.272_292del (p.Val91_Ala97del)	I/F Del	73	4774/ 282536 (41 hom)	NA	NA	NA	NA	NA	VUS ^R	PS3, BS1

(Continued on following page)

Table 3. (Continued) Details of other variants of interest

Gene ^a	Family ID ^b	Zygosity ^c	Variant description	Variant type ^d	Pub ^e	GnomAD frequency ^f	Splicing evaluation ^g		Missense evaluation ^h			ACMG evaluation ⁱ	
							HSF	BDGP	Pred	Ortho	Dom	Class	Evidence
	<i>PHN133, DDN51</i>	Het [^]	c.398C>T (p.Ala133Val)	Mis	73	1022/ 282816 (3 hom)	NA	NA	6/6	7/7	4/8	VUS	PS1, BS1
	PHN29	Hom	c.937-8T>A (p.Ala313_inslle*)	Splice	N	41/282788	60.22 to -6 site 89.17	0.67 to -6 site 0.81	NA	NA	NA	VUS	PM3, PP3, PP4
	PHN150	Het	c.1174+1G>A (p.Asp392?)	Splice	N	0	91.81 to 64.98	0.92 to <0.01	NA	NA	NA	LP I ^R	PVS1, PM2
	DDN61	C Het	c.1175-3C>T (p.Asp392?)	Splice	N	0	91.59 to 82.2	0.55 to 0.08	NA	NA	NA	VUS	PM2, PP3, PP4
	PHN45	Het	c.1469C>T (p.Pro490Leu)	Mis	ClinVar 1xVUS, 1xLB	5/250774	NA	NA	5/6	7/7	5/8	VUS	PM2
<i>SLC34A3</i>	PHN165, PHN245	C Het, Het	c.305-7G>A (p.Ser105?)	Splice	ClinVar 1x LB	43/281518	59.5 to N 88.45	0.28 to <0.1, N 0.35	NA	NA	NA	VUS	PM3, PP3, PP4
	PHN258	Het	c.362G>A (p.Gly121Glu)	Mis	N	1/249512	NA	NA	6/6	7/7	4/8	VUS	PM2
	DDN26	Het [^]	c.561-8G>A (p.Glu186_Arg187 insSerHis)	Splice	ClinVar 1x VUS	6/184272	7.69 to 1.3, N 9.37	0.76 to <0.1, N 0.74	NA	NA	NA	VUS	PM4, PP3
	PHN209	Het	c.756G>A (p.Gln252?)	Splice	74	562/ 247480 (2 hom)	96.91 to 86.33	0.98 to 0.23	NA	NA	NA	VUS	PS1, PP3
	PHN54	Het [^]	c.1208T>G (p.Met403Arg)	Mis	N	17/271426	NA	NA	5/6	4/7	2/8	VUS	PP3
	PHN239	C Het	c.1711C>T (p.Pro571Ser)	Mis	N	1/148960	NA	NA	2/6	7/7	NA	VUS	PM2, PP2, PP3, PP4
<i>SLC4A1</i>	PHN80	Het [^]	c.539G>A (p.Arg180His)	Mis	75	939/ 282824 (2 hom)	NA	NA	NA	5/8	NA	VUS	PS1, BS1
	DDN28	Het [^]	c.2716G>C (p.Glu906Gln)	Mis	12	322/ 282576	NA	NA	4/6	8/8	NA	VUS	PS1, BP5
<i>SLC7A9</i>	PHN95	Het	c.313G>A (p.Gly105Arg)	Mis	76	75/282378	NA	NA	6/6	6/7	6/6	Path II ^R	PS1, PS3, PS4, PM3, PP4
	PHN175	Het	c.544G>A (p.Ala182Thr)	Mis	76	727/ 282810 (2 hom)	NA	NA	3/6	6/7	5/7	LP II ^R	PS1, PS3
<i>SLC9A3R1</i>	PHN56	Het	c.902A>T (p.Asp301Val)	Mis	77	277/ 282774	NA	NA	4/6	5/7	8/10	VUS	PS1
<i>WNK4</i>	PHN243	Het	c.2080C>T (p.Gln694*)	Nons	N	6/282870	NA	NA	NA	NA	NA	VUS	PM4, BP1*
Chr8dup	DDN5	Het [^]	Ch8 (86,080,415-87,439,522) 1.4MB	L Dup	N	N	NA	NA	NA	NA	NA	VUS	PM2
Chr4dup	DDN5	Het [^]	Ch4 (79,698,698-80,259,893) 560kb	L Dup	N	N	NA	NA	NA	NA	NA	VUS	PM2

NA, not applicable.

^aGene: nucleotide and protein accession numbers are shown in Table S3.^bFamily ID: boldface type indicates possibly significant in the family; italicized type indicates variant in heterozygosity previously considered significant.^cZygosity: Hom, homozygous; Het, heterozygous; C Het, compound heterozygous; ^complex genotype.^dVariant type: Mis, missense; NonStop, stop codon substitution; Nons, nonsense; F/S Dup, frameshifting duplication; F/S Del, frameshifting deletion; L Dup, large duplication; I/F Del, inframe deletion.^ePub: prior description in publication; N, novel variant; description in ClinVar if unpublished: P, pathogenic; VUS, variant of uncertain significance; LB, likely benign.^fGnomAD frequency: frequency in the gnomAD database of "normal individuals", hom, homozygous descriptions.^gSplicing evaluation: HSF, Human Splice Finder; BDGP, Berkley Drosophila Gene Project, for both normal and variant score shown, and where appropriate N is score of novel site generated.^hMissense evaluation: Pred, fraction of predicted damaging pathogenicity scores from: SIFT, PolyPhen-2 HVAR, MutationTaster, Mutation Assessor, FATHMM, and FATHMM MKL; Ortho, fraction matching the human sequence in a multisequence alignment (MSA) of orthologs from mammals to fish; Dom, fraction matching the human sequence MSA of conserved domains, NCBI database.ⁱACMG evaluation: class, pathogenic classification based on the American College of Medical Genetics guidelines for interpretation of sequence variants: Path, pathogenic; LP, likely pathogenic; VUS, variant of uncertain significance, with subclasses shown; R, evaluation in recessive setting if found with another LP/P allele; Evidence, ACMG evidence supporting the interpretation of sequence variant classification. The evidence is classed as: PVS1, pathogenic very strong; PS, pathogenic strong; PM, pathogenic moderate; PP, pathogenic supportive (see Richards *et al.*¹⁹ for details).

Table 4. PH-negative (PHN) and DD-negative (DDN) families with variants of interest

Gene	Pedigree ID	Variant	Ethnicity (sex) ^a	Age at first stone	No. stones ^b	Stone comp ^c	ESKD (E) or eGFR, age ^d	NC ^e	U/Ca ^f	U/Ox ^g	U/pH ^h	U/Cit ⁱ	Comments ^j
Single variants													
<i>ALPL</i>	PHN23	c.1069C>T (p.Arg357Trp)	NA (M)	40 yr	Multi	CaOx	78, 48y	N	458	58	7.0	NA	
<i>ATP6V0A4</i>	DDN64	c.334C>G (p.Gln112Glu)	White (M)	-	0	-	69, 9y	Y, 5y	56	45	7.8	<64	
<i>BSND</i>	PHN255	c.673C>T (p.Gln225*)	White (F)	56 yr	1	NA	34, 57y	NA	45	163	5.5	<35	DM2
	PHN90	c.859G>T (p.Glu287*)	NA (F)	NA	NA	NA	E, 66y	NA	NA	NA	NA	NA	Acute tubular necrosis, oxalate nephropathy
<i>CLCNKB</i>	PHN212	c.782-2A>G (p.Glu261?)	NA (M)	69 yr	1	CaOx	20, 73y	NA	59	33 - 210	5.1	121	
<i>CYP24A1</i>	PHN200	c.428_430delAAG (p.Glu143del)	White (M)	2 yr	1	NA	NA	N	3.7	77	7.4	741	Hematuria 18 mo
	PHN63	c.1186C>T (p.Arg396Trp)	White (M)	4 yr	1	90% CaOx, 10% CaP	113, 4y	N	3.7	99	7.3	596	
	PHN234	c.1186C>T (p.Arg396Trp)	White (F)	NA	NA	COM	NA	NA	71	86	6.3	136	
	PHN68	c.1226T>C (p.Leu409Ser)	White (F)	5 yr	1	NA	149, 5y	N	2.8	73	7.4	1072	Hematuria
	PHN115	c.1226T>C (p.Leu409Ser)	White (F)	4 mo	6	NA	146, 4m	N	13	18	7.3	53	Premature
	PHN120	c.1385G>A (p.Cys462Tyr)	So Asia (?)	NA	Multi	NA	NA	N	NA	NA	NA	NA	Gross hematuria, 6 mo
<i>HNF4A</i>	PHN71	c.427A>G (p.Ser143Gly)	White (F)	NA	~100	NA	23, 63y	Y, 61y	41	84	5.9	357	
<i>KCNJ1</i>	DDN46	c.932G>A (p.Arg311Gln)	NA (M)	NA	NA	NA	55, 33y	Y, 29y	445	69	7.5	302	DI, hyperparathyroidism
<i>SLC12A3</i>	PHN249	c.1963C>T (Arg665Cys)	White (F)	NA	NA	NA	NA	Y, 1y	0.9	144	7.1	1104	
<i>SLC22A12</i>	DDN50	c.1301G>A (p.Arg434His)	Mid East (M)	9 yr	2	NA	97, 9y	N	30 mg/g	NA	7	NA	VATER syndrome
	PHN77	c.1301G>A (p.Arg434His)	White (M)	-	0	-	134, 7y	N	2.7	67	6.9	653	Gross hematuria
<i>SLC26A1</i>	PHN228	c.528C>A (p.Tyr176*)	Chinese (M)	30 yr	Multi	NA	E, 56y	NA	NA	NA	NA	NA	
<i>SLC3A1</i>	PHN136	c.1400T>C (p.Met467Thr)	White (M)	-	0	-	E, 60y	N	NA	NA	NA	NA	Kidney biopsy, oxalate crystals
<i>SLC34A1</i>	PHN88	c.115C>T (p.His39Tyr)	So Asia (M)	2 yr	10	CaOx	46, 11y	Y	<12.7 mg/g	61	NA	15.6 mg/1.73m²/24hr	Small kidneys, LVD
	PHN150	c.1174+1G>A (p.Asp392?)	White (M)	3 yr	NA	CaOx/UA	26, 65y	N	130	44	6.0	373	DM2, atrophic LK
	PHN45	c.1469C>T (p.Pro490Leu)	NA (M)	3 mo	Mult	NA	NA	N	NA	164 mg/g cr	NA	NA	Twin with stones did not have variant
	DN-21	c.272_292del (p.Val91_Ala97del)	NA (F)	54 yr	Mult	COM	25, 58y	N	56	111	6.0	NA	Ox crystals, Sjogren's syndrome
	PHN222	c.272_292del (p.Val91_Ala97del)	White (M)	50 yr	Mult	CaOx	58, 66y	N	162	73	7.0	1108	
	PHN179	c.272_292del (p.Val91_Ala97del)	White (F)	10 yr	>100	50%COM 50%UAD	NA	N	2.3	42.5	5.9	404	
<i>SLC34A3</i>	PHN165	c.305-7G>A (p.Ser105?)	White (F)	8 mo	1	NA	95, 8 mo	N	2.8	149	7.5	1363	
	PHN258	c.362G>A (p.Gly121Glu)	NA (F)	2 yo	6	NA	NA	Y, 2y	53 mg/g cr	4 mg/g cr	6.5	90	Dysmorphic features, BRS, kidney cysts
	PHN209	c.756G>A (p.Gln252?)	White (F)	14 yr	3	AP	NA	N	111	75	7.6	135	Developmental delay, Lennox-Gastaut syndrome
<i>SLC7A9</i>	PHN95	c.313G>A (p.Gly105Arg)	White (F)	1 mo	2	NA	91, 9 mo	Y, 1m	NA	142 mg/g cr	NA	NA	VSD, choreoathetosis

(Continued on following page)

Table 4. (Continued) PH-negative (PHN) and DD-negative (DDN) families with variants of interest

Gene	Pedigree ID	Variant	Ethnicity (sex) ^a	Age at first stone	No. stones ^b	Stone comp ^c	ESKD (E) or eGFR, age ^d	NC ^e	U/Ca ^f	U/Ox ^g	U/pH ^h	U/Cit ⁱ	Comments ^j
	PHN175	c.544G>A (p.Ala182Thr)	NA (M)	51 yr	Multi	CaOx	72, 62 yr	N	120	68	5.3	646	Cystine -ve
<i>SLC9A3R1</i>	PHN56	c.902A>T (p.Asp301Val)	NA (M)	13 yr	Mult	NA	NA	N	<u>0.73</u>	53 mg/g cr	5.6	251.7	
<i>WINK4</i>	PHN243	c.2080C>T (p.Gln694*)	Hisp (M)	7 yr	3	NA	124, 7 yr	NA	<u>7.8</u>	<u>51.8</u>	6.4	<u>433</u>	
Pedigree ID	Gene	Variant	Ethnicity (sex)	Age at first stone	No. stones	Stone comp	ESKD (E) or eGFR, age ^e	NC	U/Ca	U/Ox	U/pH	U/Cit	Comments
Multiple variants													
PHN280	<i>ALPL</i>	c.1001G>A (p.Gly334Asp)	White (M?)	4 yr	NA	NA	-	Y, 4.5 yr	<u>4.4</u>	<u>103.1</u>	7.3	<u>1483</u>	
	<i>SLC4A1</i>	c.706T>G (p.Phe236Val)											
PHN99	<i>ATP6V1B1</i>	c.1155dupC (p.Ile386Hisfs)	White (M)	52 yr	7	NA	72, 62 yr	NA	96	231	7.3	758	Cystine -ve
	<i>SLC3A1</i>	c.1400T>C (p.Met467Thr)											
PHN54	<i>ATP6V1B1</i>	c.181C>T (p.Gln61*)	NA (M)	12 yr	1	COM	122, 12 yr	N	<u>3.6</u>	<u>104</u>	6.5	<u>651</u>	
	<i>SLC34A3</i>	c.1208T>G (p.Met403Arg)											
PHN144	<i>CYP24A1</i>	(Hom) c.470G>A (p.Arg157Gln)	White (F)	12 yr	>100	CaOx	E, 56 yr	Y, 12 yr	NA	NA	NA	NA	MSK
PHN237	<i>CYP24A1</i>	c.470G>A (p.Arg157Gln)	White (M)	20	>300	COM	66, 62 yr	NA	258	84	5.6	2319	
	<i>SLC3A1</i>	c.161delC (p.Gln55Argfs)											
PHN80	<i>CYP24A1</i>	c.964G>A (p.Glu322Lys)	White (F)	4 yr	5	NA	171, 6 yr	N	<u>6.4</u>	<u>101</u>	7.0	<u>1165</u>	
	<i>SLC4A1</i>	c.539G>A (p.Arg180His)											
DDN48	<i>CYP24A1</i>	c.1339dupA (p.Ile447Asnfs)	Hisp (M)	-	NA	-	55, 16 yr	Y, 6 yr	<u>40 mg/g</u>	<u>60</u>	7.0	NA	Pyelonephritis
	<i>BSND</i>	c.770A>G (p.Gln257Arg)											
PHN157	<i>HNF4A</i>	c.724G>A (p.Val242Met)	NA (M)	65 yr	1	COM	79, 65 yr	N	237	81	5.8	385	
	<i>CYP27B1</i>	c.1378delC (p.Leu460Trpfs)											
PHN133	<i>SLC12A3</i>	c.363G>C (p.Glu121Asp)	White (M)	NA	Multi	COM	77, 56 yr	N	320	46	6.8	770	
	<i>SLC34A1</i>	c.398C>T (p.Ala133Val)											
PHN29	<i>SLC34A1</i>	(Homo) c.937-8T>A (p.Ala313_insls*)	N Africa (M)	-	0	-	150, 3 mo	Y, 3 mo	<u>NA</u>	<u>147 mg/g</u>	NA	NA	
DDN5	<i>Chr8dup</i>	Ch8 (86,080,415-87,439,522) 1.4MB	White (M)	-	NA	-	192, 15 yr	N	<u>2.8</u>	NA	6	NA	
	<i>Chr4dup</i>	Ch4 (79,698,698-80,259,893) 560kb											
	<i>APRT</i>	c.541T>C (p.*181Argext*)											

Biochemical values outside of the normal range are shown in boldface type.

DD, Dent disease; NA, information not available; PH, primary hyperoxaluria.

^aEthnicity (sex): So, south; Hisp, Hispanic; N, north; Mid, middle; (F), female; (M), male.

^bNo. stones, total number of stones observed; Multi, multiple.

^cStone comp, stone composition; CaOx, calcium oxalate; CaP, calcium phosphate; AP, apatite; COM, calcium oxalate monohydrate; UA, uric acid; UAD, uric acid dihydrate.

^dESKD, eGFR, age: E, end-stage kidney disease with age indicated, eGFR, value and age indicated; eGFR calculated with Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (ml/min per 1.73 m²) or full age spectrum (FAS) pediatric equation for patients <1 yr.

^eNC, nephrocalcinosis; Y, yes and age first detected; N, no.

^fU/Ca, urine calcium, shown as mg/24 h when ≥18 yr or as mg/kg per 24 h when <18 yr (underlined), unless otherwise shown.

^gU/Ox, urine oxalate, shown as mg/24 h when ≥18 yr or as mg/1.73 m² when <18 yr (underlined), unless otherwise shown.

^hU/pH, urine pH.

ⁱU/Cit, urine citrate, shown in mg/24 h when ≥18 yr or as mg/g creatinine when <18 yr (underlined). Creatinine normalization (mg/g creatinine).

^jComments: BRS, blepharophimosis renal syndrome; DI, diabetes insipidus; DM2, diabetes mellitus; LK, left kidney; LVD, left ventricular dysfunction; MSK, medullary sponge kidney; Ox, oxalate; VSD, ventricular septal defect.

phosphorus in these individuals. Thus, it is possible that other genetic changes are contributing to the phenotypes.

Only 1 in 11 *CYP24A1* monoallelic cases had the characteristic *CYP24A1* deficiency phenotype, and so 10 were classified as subjects with a variant of interest (Table 4).⁶¹ Of interest, 4 monoallelic cases had an additional novel atypical splicing or missense change in the same gene; however, lack of data showing disrupted splicing, phase of the variants, and/or significance of the substitution resulted in their classification as a VUS in each case. An example is the *CYP24A1* variant, p.Arg157Gln,⁷³ which was difficult to evaluate even though *in vitro* analysis previously demonstrated reduced expression.⁷³ A different substitution at the same site, p.Arg157Trp,⁴¹ is an accepted pathogenic variant, and a subject with these 2 variants *in trans* had a typical 24-hydroxylase deficiency phenotype (DDN51), whereas a patient homozygous for p.Arg157Gln (PHN144) did not (Tables 1, 4). Interestingly, DDN51 also has the *SLC34A1*: p.Ala133Val variant. We classified DDN51 as a monoallelic patient but considered that p.Arg157Gln may have a modifying role.⁷³

Although USD is less common among children than in adults, the diagnosis of USD and NC in the pediatric age group has been rising.^{93,94} These pediatric cases are highly enriched for monogenic causes,⁸⁸ and the majority (76.1%) of our genetically resolved cases were first diagnosed with USD/NC before the age of 18 years, a higher proportion than in the total cohort, emphasizing the enrichment of monogenic disease in pediatric cases.⁸⁸ However, more surprisingly, we did not see a different representation of children/adolescents in biallelic (78%) versus monoallelic (78%) subjects, as a milder course is often characteristic of monoallelic disease.⁵⁷ In the biallelic cohort, 19 of 27 (70.4%) were homozygous, indicating the importance of consanguinity for enrichment of certain diseases. The claudin-related diseases are a good example, with unique or very rare variants accounting for most cases. However, for some genes, such as for *CYP24A1*, homozygosity in an outbred population is not unusual because of the high population levels of some alleles. For other gene alleles, such as *SLC34A1*: p.Tyr489Cys, a rarer variant can become enriched in specific populations, Icelandic in this case.⁸ Analysis of an adult population with limited consanguinity would be expected to yield fewer monogenic cases.^{93,94}

Despite the interesting yield and breadth of causes in the newly resolved cases, a majority of the entire cohort remained unresolved after this further tNGS analysis. It is likely that many individuals do not have simple monogenic disease, because there is significant phenotypic overlap with typical USD, in which genetic

risk factors are important but not singly causative. However, follow-up studies of newly detected VUS, with, for instance, further family analysis, may resolve additional cases, as may a broader genetic screen such as WES or whole genome sequencing (WGS) if larger pedigrees are available. Excluding known USD/NC genes, as we have done, is also a key step before novel monogenic causes of USD/NC can be identified, with multiplex families especially helpful for these next-step studies.

Our study has certain limitations. The study was retrospective in nature, and for some individuals we lacked detailed clinical information at the time of genetic testing, often due to the local unavailability of particular biochemical tests. Thus, the evidence of PH or DD was sometimes limited and upon retrospective review in a small minority inconsistent with the initially suspected diagnosis. Nevertheless, this mirrors the situation in clinical practice, as detailed biochemical data may not always be available, especially when a patient presents in kidney failure. Furthermore, this cohort was assembled over a relatively long period, with recent data often missing. Recruitment for this study focused on the patient, and so only a minority had samples and clinical information from family members, limiting segregation analysis. By design, use of a candidate gene panel rather than WES or WGS limited novel gene discovery, although several candidate genes were included on the panel, and since performing this study additional USD genes have been identified that were not included on our panels. Because of the populations from which the cohort was recruited, the whole range of monogenic USD/NC was not evenly represented, with, for instance, higher urinary excretions of oxalate (from the PH-suspected cohort) or LMWP (from the DD group) than is typical overall among USD/NC cases. Finally, in some instances, the effect of missense variants on protein function was not certain, and *in vitro* evaluation of these variants would be of value, even if these studies also need to be rigorously assessed.

In conclusion, our genetic rescreening of the cohort of patients initially suspected to have PH or DD resolved an additional 13.1% of these cases, and a variety of monoallelic and biallelic variants in 14 genes were implicated. Given the phenotypic overlap of monogenic causes of USD and NC, a tNGS approach is a cost-effective and efficient way to resolve cohorts suspected of monogenic disease.

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SUPPLEMENTARY MATERIAL

[Supplementary File \(PDF\)](#)

Supplementary Methods

Supplementary References

Figure S1. DDN26 has an infragenic duplication of *SLC34A1*

Table S1. Genes on the 90 gene and 102 gene panels

Table S2. Details of novel Sanger detected PH and DD gene pathogenic variants

Table S3. Genes with transcript and protein accession numbers

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