ORIGINAL ARTICLE

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Molecular Diagnoses of X-Linked and Other Genetic Hypophosphatemias: Results From a Sponsored Genetic Testing Program

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ABSTRACT

X-linked hypophosphatemia (XLH), a dominant disorder caused by pathogenic variants in the *PHEX* gene, affects both sexes of all ages and results in elevated serum fibroblast growth factor 23 (FGF23) and below-normal serum phosphate. In XLH, rickets, osteomalacia, short stature, and lower limb deformity may be present with muscle pain and/or weakness/fatigue, bone pain, joint pain/stiffness, hearing difficulty, enthesopathy, osteoarthritis, and dental abscesses. Invitae and Ultragenyx collaborated to provide a no-charge sponsored testing program using a 13-gene next-generation sequencing panel to confirm clinical XLH or aid diagnosis of suspected XLH/other genetic hypophosphatemia. Individuals aged ≥6 months with clinical XLH or suspected genetic hypophosphatemia were eligible. Of 831 unrelated individuals tested between February 2019 and June 2020 in this cross-sectional study, 519 (62.5%) individuals had a pathogenic or likely pathogenic variant in *PHEX* (*PHEX*-positive). Among the 312 *PHEX*-negative individuals, 38 received molecular diagnoses in other genes, including *ALPL*, *CYP27B1*, *ENPP1*, and *FGF23*; the remaining 274 did not have a molecular diagnosis. Among 319 patients with a provider-reported clinical diagnosis of XLH, 88.7% ($n = 283$) had a reportable *PHEX* variant; 81.5% ($n = 260$) were *PHEX*-positive. The most common variant among *PHEX*-positive individuals was an allele with both the gain of exons 13–15 and c.*231A>G (3′UTR variant) ($n = 66/519$). Importantly, over 80% of copy number variants would have been missed by traditional microarray analysis. A positive molecular diagnosis in 41 probands (4.9%; 29 *PHEX* positive, 12 non-*PHEX* positive) resulted in at least one family member receiving family testing. Additional clinical or family member information resulted in variant(s) of uncertain significance (VUS) reclassification to pathogenic/likely pathogenic (P/LP) in 48 individuals, highlighting the importance of segregation and clinical data. In one of the largest XLH genetic studies to date, 65 novel *PHEX* variants were identified and a high XLH diagnostic yield demonstrated broad insight into the genetic basis of XLH. © 2021 The Authors. *Journal of Bone and Mineral Research* published by Wiley Periodicals LLC on behalf of American Society for Bone and Mineral Research (ASBMR).

KEY WORDS: GENETIC RESEARCH - HUMAN ASSOCIATION STUDIES; DISORDERS OF CALCIUM/PHOSPHATE METABOLISM- OTHER; DISEASES AND DISORDERS OF/RELATED TO BONE - OTHER; THERAPEUTICS – OTHER; CELL/TISSUE SIGNALING - PARACRINE PATHWAYS – OTHER

Received in original form June 5, 2021; revised form September 27, 2021; accepted October 2, 2021.
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Journal of Bone and Mineral Research, Vol. 37, No. 2, February 2022, pp 202–214.
DOI: 10.1002/jbmr.4454

Introduction

X-linked hypophosphatemia (XLH) is a rare X-linked dominant genetic disorder of renal phosphate wasting that affects male and female individuals of all ages,[1] with an estimated prevalence of 1 in 20,000 to 25,000 individuals.[2-5] XLH causes varying clinical features, including rickets, osteomalacia, short stature, lower limb deformity, muscle pain, weakness, fatigue, bone pain, joint pain or stiffness, hearing difficulty, enthesopathy, osteoarthritis, and dental abscesses.[6] The disorder is caused by pathogenic variants in the *PHEX* gene,[7] which lead to elevated (or inappropriately normal) serum fibroblast growth factor 23 (FGF23) levels and ultimately below-normal serum phosphate levels.[8]

XLH is the most common form of hypophosphatemic rickets,[1] distinguishable from other forms by its genetic cause, pattern of inheritance, and disease management.[8,9] However, it can be difficult to differentiate between the various forms of familial hypophosphatemia because they share similar signs, symptoms, and biomarkers.[9] For example, pathogenic variants in *FGF23* are associated with autosomal dominant hypophosphatemic rickets (ADHR) and result in a clinical manifestation that is strikingly similar to XLH, though symptoms associated with ADHR later in life may help differentiate between the two.[10] Further, other genetic disorders such as hypophosphatasia (HPP) show phenotypic overlap with familial hypophosphatemia but differ in serum phosphate levels and recommendations for clinical management.[11] It is therefore important to confirm the underlying molecular etiology of hypophosphatemia before initiating therapy.

In February 2019, Ultragenyx Pharmaceutical and Invitae initiated the sponsored Hypophosphatemia genetic testing program, providing no-charge genetic testing to patients to confirm a clinical XLH diagnosis or to aid diagnosis of suspected XLH or other genetic hypophosphatemia. This study was designed to describe outcomes from the Hypophosphatemia sponsored program, including diagnostic yield, genetic landscape of detected *PHEX* variants, and clinical characteristics of all individuals in the cohort. Although the results of genetic testing typically focus on the proband, this program also supported cascade testing in identified at-risk relatives, which resulted in additional diagnoses and greatly assisted in variant reclassification.

PATIENTS AND METHODS

Study population

In this cross-sectional study, individuals referred for genetic testing through the sponsored Hypophosphatemia program following clinical assessment between February 27, 2019, and June 30, 2020, were included. Individuals were eligible if they were 6 months or older and (i) had a clinician-completed start form for a new treatment for XLH (burosumab; a human monoclonal antibody directed against FGF23), which served as an indication of a clinical diagnosis of XLH; (ii) had a previous clinical diagnosis related to hypophosphatemia; or (iii) was suspected to have genetic hypophosphatemia based on the presence of two or more clinical signs or symptoms. When hypophosphatemia was suspected, ordering providers were asked to report the following signs and symptoms on the requisition form: family history of confirmed XLH; muscle pain, weakness, and/or fatigue; lower limb deformities; fractures/pseudo-fractures; tooth abscesses and/or excessive dental caries; bone pain, joint pain, and/or joint stiffness; short stature; and gait abnormalities.

Genetic testing and variant interpretation

Individuals provided blood or saliva samples and were tested using next-generation sequencing (NGS) with a multi-gene panel consisting of 13 genes that were selected based on associations with hypophosphatemia as reported in the medical literature: *ALPL*,[12-18] *CLCN5*,[19-22] *CYP2R1*,[23-26] *CYP27B1*,[27-30] *DMP1*,[31-38] *ENPP1*,[39-42] *FAH*,[43-45] *FAM20C*,[46-52] *FGF23*,[10,53-59] *FGFR1*,[60-63] *PHEX*,[64-68] *SLC34A3*,[69-71] and *VDR*.[72,73] Each gene in the NGS panel was targeted with oligonucleotide baits (Agilent Technologies, Santa Clara, CA, USA; Roche, Pleasanton, CA, USA; IDT, Coralville, IA, USA) that were designed to capture exons, the 10 bases flanking exonic sequences, and certain non-coding regions of interest (*PHEX* c.*231A>G). Genes were sequenced at a high-depth coverage (20× minimum, 150× average). A bioinformatics pipeline that incorporates community standard and custom algorithms was used to analyze NGS reads and identify single nucleotide variants (SNVs), small and large insertions/deletions (indels), structural variants, and exon-level copy number variants (CNVs).[74,75] In particular, CNVs were detected using split-read analysis and copy number counting method that uses a statistical mixture model for sequence read counts within target regions (typically exons), and employs model-based segmentation algorithms optimized for use with sparsely distributed targets across the genome.[75,76] This method estimates the most likely copy number for all segments, and critically for clinical use, each called segment is assigned a robust quality score indicating confidence in the copy number determination.

All identified variants were interpreted according to the joint consensus from the American College of Medical Genetics and Genomics and the Association for Molecular Pathology using Invitae's proprietary point-based scoring system, Sherloc.[77,78] The following transcripts were used to interpret variants: *ALPL* NM_000478.5, *CLCN5* NM_000084.4, *CYP2R1* NM_024514.4, *CYP27B1* NM_000785.3, *DMP1* NM_004407.3, *ENPP1* NM_006208.2, *FAH* NM_000137.2, *FAM20C* NM_020223.3, *FGF23* NM_020638.2, *FGFR1* NM_023110.2, *PHEX* NM_000444.5, *SLC34A3* NM_080877.2, and *VDR* NM_001017535.1. Following interpretation, variants were classified as pathogenic (P), likely pathogenic (LP), variant(s) of uncertain significance (VUS), likely benign (LB), or benign (B). Variants classified as P, LP, and VUS were reported to the ordering clinician. P/LP variants were considered a positive molecular diagnosis. In addition, a variant was categorized as novel if it met the following three criteria: (i) had never been reported in ClinVar by a clinical testing laboratory (excluding the testing laboratory for this study, Invitae), (ii) had never been reported in published literature, and (iii) had never been observed by Invitae in individuals tested outside of the sponsored testing program.

Cascade testing

Individuals who were the first in their family to be tested through the sponsored testing program were considered probands. At clinician discretion, cascade testing could be offered to at-risk relatives of a proband with a positive result. These relatives were also eligible to be tested through the Hypophosphatemia sponsored program, and their data were analyzed separately in this study. The laboratory also recommended family testing when VUS in *PHEX* were identified and additional clinical information

or segregation analysis could aid in reclassification. The outcomes of VUS reclassification were reported to ordering clinicians.

Data analysis

In all analyses, data were summarized using descriptive statistics. Demographic characteristics included age, sex, and ancestry. In reporting on the clinical signs and symptoms, calculations were based on the number of individuals for whom the sign or symptom was reported by the ordering clinician. A clinical diagnosis of hypophosphatemia was considered if either the treatment start form had been completed or the ordering physician had indicated that a clinical diagnosis had been made.

The diagnostic yield was calculated for those with a molecular diagnosis in *PHEX* (ie, "*PHEX* positive;" one P/LP variant) and those with a molecular diagnosis in a gene other than *PHEX* (ie, "non-*PHEX* positive;" monoallelic for autosomal dominant or X-linked diseases and biallelic for autosomal recessive diseases). Of note, HPP (caused by pathogenic variants in *ALPL*) could be autosomal recessive or autosomal dominant. Demographics were compared based on genetic testing results: *PHEX* positive, non-*PHEX* positive, and no molecular diagnosis (ie, no P/LP variant for autosomal dominant or X-linked diseases; zero or one P/LP variant for an autosomal recessive diseases).

The number of individuals with each clinician-reported sign or symptom was calculated and stratified by molecular diagnosis. The contribution of a history of XLH or signs and symptoms to a positive molecular diagnosis was also explored. For this analysis, probands were categorized into three groups: (i) those who had a family member with a confirmed XLH diagnosis or had a previous hypophosphatemia-related diagnosis of their own, and who had reported signs and symptoms; (ii) those who had a personal or family history but no reported signs or symptoms; and (iii) those who did not have a personal or family history but had reported signs or symptoms.

Diagnostic yield and demographic information were evaluated separately among family members seeking cascade testing following a proband's positive test result in *PHEX* or another gene on the panel. In addition, the number of families who underwent testing and the mean number of family members tested per proband were calculated. Among family members who underwent testing through the sponsored program (for whom clinical symptoms were reported on the sponsored program's requisition form), reported clinical features were summarized when available.

Statistical analysis

A one-way ANOVA assessed differences in age based on genetic test result (ie, *PHEX*-positive, non-*PHEX*-positive, negative). Based on the ANOVA results, a Tukey post hoc test evaluated differences in age among the three groups. Values of $p \leq 0.05$ were considered statistically significant.

Results

Genetic testing results among probands

During the study period, 831 unrelated individuals (probands) received testing through the sponsored Hypophosphatemia program. Age at time of testing among probands ranged from 6 months to 79 years (mean $\pm$ standard deviation [SD], 26.9 $\pm$ 20.1 years) (Table 1). The majority of individuals were female (65.9%) and of self-reported White ancestry (57.0%).

In total, 237 unique P/LP *PHEX* variants were reported in 519 individuals (62.5%), three of whom also carried a VUS in *PHEX*. In addition, 49 *PHEX* VUS were detected in 50 individuals (6.0%) (Fig. 1, Supplemental Table S1). In seven *PHEX*-positive individuals (1.3%), an additional P/LP variant was detected in another gene, including *ALPL* ($n = 1$), *CYP27B1* ($n = 3$), *FAH* ($n = 1$), and *SLC34A3* ($n = 2$). The additional P/LP findings were associated with a molecular diagnosis in one individual (*ALPL*) and carrier status for an autosomal recessive disorder in the other six individuals, two of whose P/LP variants were in *SLC34A3*, which has been previously reported to be associated with hypercalciuria in some carriers.[69,79,80]

Of the 312 cases (37.5%) in which no *PHEX* molecular diagnosis was found, 38 individuals had molecular diagnoses in four genes associated with other disorders (Fig. 1). In this group, the most common gene with a positive molecular diagnosis was *ALPL* ($n = 31$), which is associated with HPP; 27 probands had a single P/LP variant associated with autosomal dominant HPP and the remaining four carried two P/LP variants associated with autosomal recessive HPP. Among the remaining seven individuals with non-*PHEX*-positive molecular diagnoses, four had one P/LP variant in *FGF23* (autosomal dominant hypophosphatemic rickets), two were biallelic for *CYP27B1* (autosomal recessive vitamin D dependent rickets), and one was biallelic for *ENPP1* (autosomal recessive hypophosphatemic rickets type 2) (Fig. 1). In addition, nine individuals were identified as monoallelic for an autosomal recessive disorder. Of note, five individuals were monoallelic for *SLC34A3*, which is known to result in mild symptoms such as hypercalciuria, renal stones, and nephrocalcinosis in approximately one-quarter of heterozygotes.[79] In total, 39 unique P/LP variants and 88 unique VUS were identified in non-*PHEX* genes (Supplemental Table S2).

In comparing demographic characteristics of individuals based on molecular diagnosis (Table 1), age differed significantly between the groups (one-way ANOVA, $F[2,830] = 10.43$, $p < 0.0001$). A Tukey post hoc test revealed that mean age at time of testing was significantly lower in *PHEX*-positive individuals than in those with a non-*PHEX* positive result ($p < 0.05$). However, there was no difference in mean age at testing between those with no molecular diagnosis and those with a *PHEX*-positive or non-*PHEX*-positive diagnosis. Across all diagnostic groups, the majority of individuals were female (*PHEX*-positive 66.5%, non-*PHEX*-positive 71.1%, negative 64.2%) and of self-reported White ancestry, though the proportion of White individuals was highest among those with a non-*PHEX* molecular diagnosis (*PHEX*-positive 54.7%; non-*PHEX*-positive 81.6%; negative 58.0%).

Clinical signs and symptoms

Several similarities and differences exist in which clinical signs and symptoms were reported based on genetic test results (*PHEX*-positive, non-*PHEX*-positive, negative) (Table 1). Reduced tubular maximum reabsorption of phosphate/glomerular filtration rate (TmP/GFR) (4.6%, 2.6%, 6.6%, respectively) and gait abnormalities (25.0%, 26.3%, 20.4%, respectively) were reported at similar rates across all groups. Hypophosphatemia-related clinical diagnoses (87.3%, 39.5%, 53.6%, respectively) were most commonly reported in *PHEX*-positive individuals, whereas tooth abscesses and/or excessive dental caries (14.8%, 36.8%, 17.5%, respectively) and fractures and/or pseudofractures (11.9%,

Table 1. Proband Demographic and Clinical Characteristics

	PHEX+ ($n = 519$)	Non-*PHEX*+ ($n = 38$)	Negative ($n = 274$)	Total ($n = 831$)
Sex, *n* (%)				
Female	345 (66.5)	27 (71.1)	176 (64.2)	548 (65.9)
Male	174 (33.5)	11 (28.9)	98 (35.8)	283 (34.1)
Age (years)				
Mean ± SD	24.4 ± 19.1	31.4 ± 19.5	30.8 ± 21.2	26.8 ± 20.1
Min, max	0.5, 79	1, 72	0.5, 78	0.5, 79
Ethnicity, *n* (%)[a]				
White	284 (54.7)	31 (81.6)	159 (58.0)	474 (57.0)
Hispanic	81 (15.6)	0	45 (16.4)	126 (15.2)
Black	47 (9.1)	0	20 (7.3)	67 (8.1)
Asian	8 (1.5)	1 (2.6)	5 (1.8)	14 (1.7)
Other	11 (2.1)	1 (2.6)	5 (1.8)	17 (2.0)
Multiple ancestries	15 (2.9)	2 (5.3)	11 (4.0)	28 (3.4)
Unknown	73 (14.1)	3 (7.9)	29 (10.6)	105 (12.6)
Reported clinical feature, *n* (%)				
Reduced TmP/GFR	24 (4.6)	1 (2.6)	18 (6.6)	43 (5.2)
Reduced serum phosphate	167 (32.2)	7 (18.4)	72 (26.3)	246 (29.6)
Tooth abscesses/excessive dental caries	77 (14.8)	14 (36.8)	48 (17.5)	139 (16.7)
Fractures/pseudofractures	62 (11.9)	17 (44.7)	70 (25.5)	149 (17.9)
Family member of a confirmed XLH patient	130 (25.0)	3 (7.9)	47 (17.2)	180 (21.7)
Gait abnormalities	130 (25.0)	10 (26.3)	56 (20.4)	196 (23.6)
Muscle pain/weakness/fatigue	105 (20.2)	16 (42.1)	122 (44.5)	243 (29.4)
Lower limb deformities	190 (36.6)	4 (10.5)	76 (32.5)	270 (32.5)
Bone or joint pain/joint stiffness	128 (24.7)	20 (52.6)	134 (48.9)	282 (33.9)
Short stature	188 (36.2)	9 (23.7)	182 (66.4)	289 (34.8)
Hypophosphatemia-related clinical diagnosis	453 (87.3)	15 (39.5)	147 (53.6)	615 (74.0)

Q1 = first quartile; Q3 = third quartile.
[a]Ethnicity was self-reported by the individual or caregiver.

44.7%, 25.5%, respectively) were more commonly reported in non-*PHEX*-positive individuals and short stature (36.2%, 23.7%, 66.4%, respectively) was most common in individuals without a diagnosis. Individuals with a *PHEX* VUS had similar clinical features reported to those who were *PHEX*-positive, with the exception of fewer reports of family members with a confirmed XLH diagnosis or a hypophosphatemia-related clinical diagnosis (Supplemental Table S3).

A hypophosphatemia-related clinical diagnosis was reported in nearly three-quarters of all probands ($n = 615/831$, 74.0%) and was strongly associated with a positive molecular diagnosis (Fig. 2*A*). Among the 319 individuals with a confirmed XLH

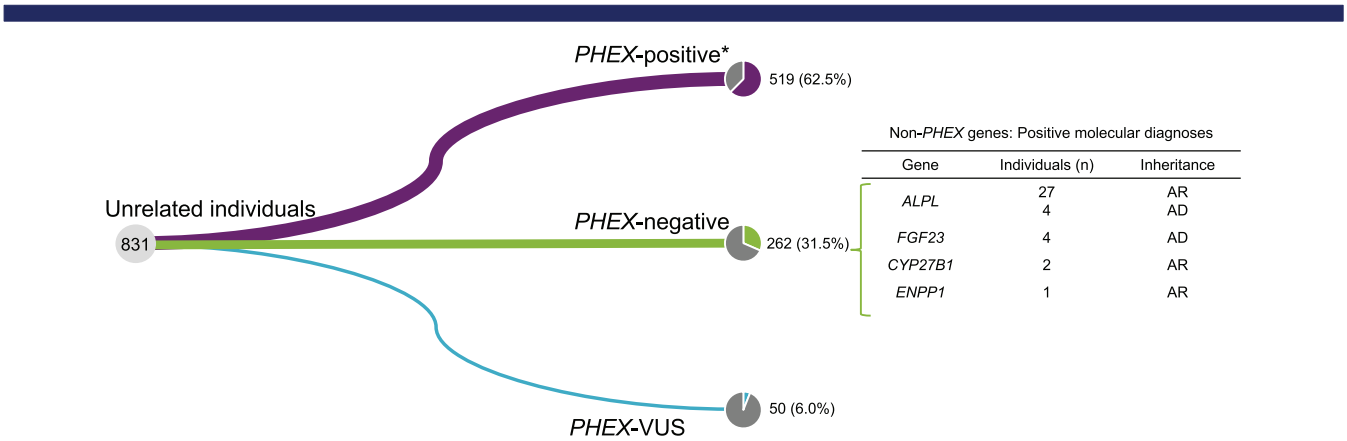

Fig 1. *PHEX* and non-*PHEX* results from probands. Probands were categorized according to their genetic testing results - *PHEX* positives (1 P/LP in *PHEX*). Among PHEX-negative individuals, those with a positive molecular diagnosis in another gene are indicated. A positive molecular diagnosis was based on the inheritance pattern (AR, 2 P/LP variants; AD, 1 P/LP variant). *Three *PHEX*-positive individuals also carried a *PHEX* VUS and were only counted in the *PHEX*-positive group. In addition, one *PHEX*-positive individual had a second molecular diagnosis in *ALPL*, with a single P/LP resulting in the AD form of the disorder. AD = autosomal dominant; AR = autosomal recessive; P/LP = pathogenic/likely pathogenic; VUS = variant of uncertain significance.

diagnosis (had a clinician-completed treatment start form for burosumab), 283 (88.7%) had a reportable P/LP variant or VUS in *PHEX* and 81.5% (260/319) had a molecular diagnosis. In total, 501 probands had at least one sign or symptom reported (range, one to eight), with short stature (34.8%); bone, joint pain, and/or joint stiffness (33.9%); lower limb deformities (32.5%); and muscle pain, weakness, and/or fatigue (29.2%) being the most common (Fig. 2*A*). However, the signs and symptoms most commonly associated with a positive *PHEX* molecular diagnosis were a family member with a confirmed XLH diagnosis (73.7%), lower limb deformities (70.4%), and reduced serum phosphate (67.9%) (Fig. 2*A*). None of the reported symptoms were strongly associated with a positive finding in a non-*PHEX* gene, with the most common features including fractures or pseudofractures (11.4%) and tooth abscesses and/or excessive dental caries (10.1%). A past hypophosphatemia-related diagnosis or a family member with a confirmed XLH diagnosis was the strongest indicator of a *PHEX*-positive molecular diagnosis, regardless of whether symptoms were reported (Fig. 2*B*). Family history was not reported for the majority of individuals without a molecular diagnosis and only reported signs or symptoms associated with hypophosphatemia (Fig. 2*B*).

Distribution and characterization of *PHEX* variants

Of the 237 unique P/LP *PHEX* variants, SNVs represented the most common variant type ($n = 89$, 37.6%) followed by small deletions, duplications, and insertions ($n = 59$, 24.9%) (Fig. 3*A*). Among all detected non-unique *PHEX* variants ($n = 585$), SNVs (46.3%) and CNVs (24.3%) were the most commonly detected variant types (Fig. 3*B*). Among CNVs ($n = 142$), a minimum of 81.0% would have been missed had microarray assays been

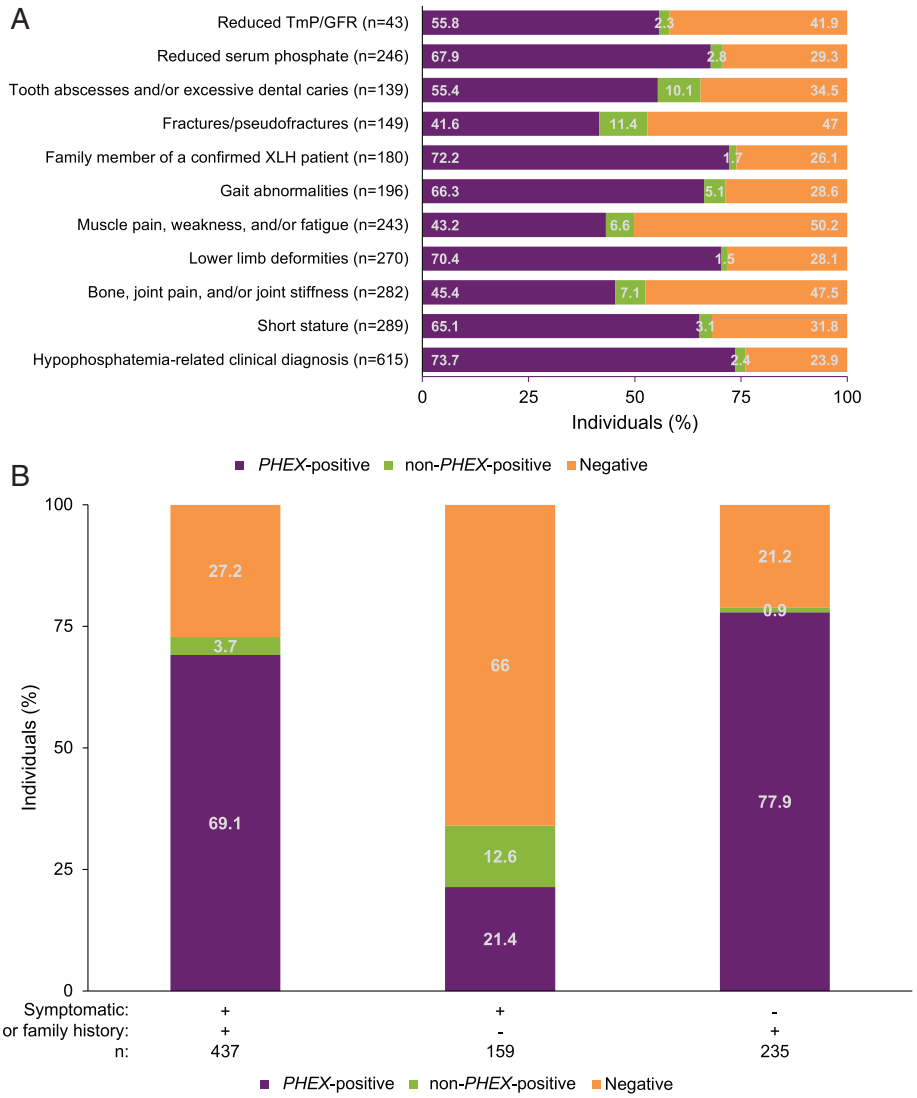

Fig 2. Diversity of reported clinical features among unrelated individuals. (*A*) The number of individuals with each clinician-reported sign or symptom was calculated and stratified by molecular diagnosis, with proportions based on the number of individuals with each feature noted. (*B*) For each clinician-reported sign or symptom, the proportions of *PHEX*-positive and non-*PHEX*-positive individuals were calculated. Proportions were calculated based on the number of individuals with the clinical sign or symptom noted ("+") or not noted ("–"), as indicated in parentheses along the X-axis.

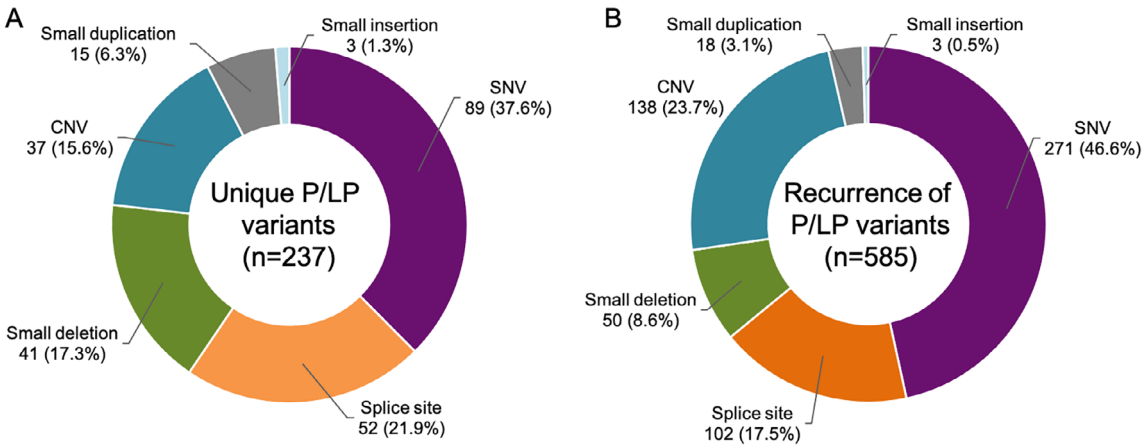

Fig 3. Distribution of *PHEX* P/LP variants observed in probands. (*A*) Distribution of variant types among the 237 unique P/LP variants. (*B*) Recurrence of *PHEX* P/LP variants by type observed across all individuals with a positive *PHEX* molecular diagnosis ($n = 585$). Note that individuals ($n = 65$) with the exon 13–15 duplication and c.*231G>A 3′UTR variants *in cis* were counted in both the CNV and SNV categories. CNVs were defined as >100 base pairs. Small deletions, duplications, and insertions were defined as events involving <100 base pairs. CNV = copy number variant; P/LP = pathogenic/likely pathogenic; SNV = single nucleotide variant.

used, due to their small size. The most common *PHEX* variants identified are reported in Table 2 (see Supplementary Table S1 for a list of all P/LP variants and VUS).

The most commonly observed variants were the gain of exons 13–15 and the 3′UTR c.*231A>G. As previously reported,[81] these two variants were observed together in the majority of probands ($n = 65/66$) and the majority of family members (some of whom were tested through the program and some of whom were tested at Invitae but outside of the program) ($n = 23/24$), suggesting that they constitute a single allele that co-segregates. In one case, an affected proband had the gain of exons 13–15 without the c.*231A>G variant and a family member had the c.*231A>G variant alone; however, CNV analysis had reduced sensitivity in this case due to triple X syndrome in the family member, so the presence of the duplication event in this patient is unknown. Of the 88 individuals who had both variants, 51 were confirmed to have both on the same chromosome. Phase information for the other 37 individuals is unknown. To explore the

Table 2. Pathogenic or Likely Pathogenic *PHEX* Variants Observed Four or More Times in Probands

PHEX variant	Effect	Interpretation	Number of patients
c.1405-?_1645+?dup	Gain (Exons 13–15)	Likely pathogenic	66[a]
c.*231A>G	Noncoding	Likely pathogenic	65
c.1601C>T	p.Pro534Leu	Pathogenic	19
c.2104C>T	p.Arg702*	Pathogenic	16
c.1735G>A	p.Gly579Arg	Pathogenic	15
c.1645+1G>A	Splice donor	Pathogenic	14
c.871C>T	p.Arg291*	Pathogenic	13
c.2239C>T	p.Arg747*	Pathogenic	9
c.1080-?_1302+?del	Deletion (Exons 10–11)	Pathogenic	8
c.1699C>T	p.Arg567*	Pathogenic	8
c.-562-?_118+?del	Deletion (Exon 1)	Pathogenic	6
c.1645C>T	p.Arg549*	Pathogenic	6
c.2071-?_*3357+?del	Deletion (Exons 21–22)	Pathogenic	6
c.304G>A	p.Gly102Arg	Pathogenic	6
c.-562-?_187+?del	Deletion (Exons 1–2)	Pathogenic	5
c.1405-?_*3357+?del	Deletion (Exons 13–22)	Pathogenic	4
c.1483-1G>C	Splice acceptor	Pathogenic	4
c.1646-?_1700+?del	Deletion (Exon 16)	Pathogenic	4
c.1700+1G>A	Splice donor	Pathogenic	4
c.1848del	p.Lys616Asnfs*3	Pathogenic	4
c.2028_2032dup	p.Phe678Serfs*11	Pathogenic	4
c.2237G>A	p.Cys746Tyr	Pathogenic	4

LP = likely pathogenic; P = pathogenic.
[a]65 of the 66 individuals with the exons 13–15 gain also had the c.*231A>G non-coding change in the 3′UTR.

spectrum of phenotypes associated with these two variants, the frequency of each symptom was calculated among probands with both variants. Among the 65 probands with both a gain of exons 13–15 and the c.*231A>G variant, a previous hypophosphatemia-related clinical diagnosis (86.2%) was the most commonly reported clinical feature, followed by lower limb deformities (43.1%) and bone, joint pain, and/or joint stiffness (38.5%) (Table 3). These variants were observed together in individuals of each of White, Hispanic, and Black ancestries.

Among the 237 unique P/LP variants, 65 (27.4%) were novel by our reported criteria and are reported for the first time here (Supplementary Table S1). These variants were observed in 87 probands. The most common novel variants were the deletion of exon 1 ($n = 6$), p.Phe678Serfs*11 (c.2028_2032dup, $n = 4$), the deletion of exons 15–20 ($n = 3$), the deletion of exons 16–20 ($n = 3$), and p.Met300* (c.898del, $n = 3$). The remaining novel *PHEX* P/LP variants were observed in one or two individuals. The majority of these individuals (87.4%) had a previous hypophosphatemia-related diagnosis. The most common reported clinical features were short stature (33.3%), reduced serum phosphate (31.0%), lower limb deformities (24.1%), and gait abnormalities (21.8%). Five additional novel *PHEX* variants were classified as VUS.

Cascade testing among family members

A positive molecular diagnosis in 41 probands (4.9%; 29 *PHEX* positive, 12 non-*PHEX* positive) resulted in at least one family member receiving family testing. In total, 103 family members received genetic testing (mean, 2.5 family members per proband; range, 1 to 26).

Cascade testing for *PHEX* was pursued in 70 relatives from 29 families (mean, 2.4 family members per proband; range, 1 to 26). Among the 70 relatives who underwent cascade testing for *PHEX* (mean age, 32.4 ± 19.4 years), 40 (57.1%) received a positive molecular diagnosis in *PHEX*. One proband, who was found to have a gain of exons 13–15 and the c.*231A>G variant in *PHEX*, had 26 relatives subsequently tested. Among these relatives, symptoms varied according to clinical information available for most of them ($n = 24$). The two most common reported symptoms in this family were tooth abscesses and/or excessive dental caries ($n = 14$, 58.3%) and bone pain, joint pain, and/or joint stiffness ($n = 12$, 50.0%). Additional symptoms were reported in other family members, representing a spectrum of phenotypes.

Cascade testing for non-*PHEX* genes was pursued for 33 individuals from 12 families (mean, 2.8 family members per proband; range, 1 to 6). All but one proband had received a molecular diagnosis in *ALPL*; the remaining proband had received a molecular diagnosis in *FGF23*. Among the 33 relatives who underwent cascade testing for non-*PHEX* genes (mean age, 28.7 ± 20.5 years), 19 (57.6%) received a positive molecular diagnosis.

Table 3. Symptoms and Other Clinical Information Reported for Probands with Gain of Exons 13–15 and 3′UTR c.*231A>G Variants

Clinical information	Number (%) ($n = 65$)
Previous diagnosis related to hypophosphatemia	56 (86.2)
Lower limb deformities	28 (43.1)
Short stature	23 (35.4)
Family member of a confirmed XLH patient	22 (33.9)
Gait abnormalities	16 (24.6)
Bone, joint pain, and/or joint stiffness	25 (38.5)
Muscle pain, weakness, and/or fatigue	18 (27.7)
Tooth abscesses and/or excessive dental caries	13 (20.0)
Fractures/pseudofractures	5 (7.7)
Reduced serum phosphate	27 (41.5)
Reduced TmP/GFR (<LLN)	2 (3.1)

LLN = lower limit of normal; TmP/GFR = ratio of tubular maximum reabsorption rate of phosphate to glomerular filtration rate; XLH = X-linked hypophosphatemia.

VUS resolution

Family testing or clinical information (eg, family or personal history, laboratory data) was requested to help with reclassification of 26 VUS. Thirteen of these VUS were subsequently reclassified as P or LP due to this additional information, impacting 48 individuals (Table 4). In addition, three VUS were reclassified to P during the study period due either to the variant being observed in individuals tested outside of the program or to additional literature, impacting nine individuals tested through the program.

Discussion

In this study, we report the results from a sponsored genetic testing program in individuals who are known or suspected to have a genetic hypophosphatemia. In just over 1 year, the sponsored Hypophosphatemia program provided access to genetic testing for 831 probands—the largest cohort to date of individuals with a molecular diagnosis in *PHEX*. Although XLH has been estimated to affect 1 in 20,000 to 25,000 individuals,[2–5] based on data presented here from 831 probands and their family members, this prevalence may be underestimated.

The ability to test a large number of individuals also allows a fuller view of the genetic landscape of this condition. An analysis of the distribution of P/LP *PHEX* variants observed in this study identified 237 unique variants, 65 of which were novel. Of all P/LP *PHEX* variants observed ($n = 585$, including non-unique variants), a large proportion were CNV events (23.6%, $n = 138$). Due to the nature of the NGS assay, the full size of deletion and duplication events that include either the first or the last exon of *PHEX* cannot be determined, as these events likely extend beyond the reportable range of the gene. However, 81% of the *PHEX* P/LP CNV events detected were subgenic (ie, smaller than a full gene deletion with both *PHEX* intronic boundaries determined) and would be missed by traditional microarray analysis due to their small size. This finding highlights the importance of CNV testing with single-exon resolution (eg, NGS, multiplex ligation-dependent probe amplification) for patients with a suspected diagnosis of XLH. Though NGS panels can detect a wide range of changes at the DNA sequence level, other assays can detect a different set of abnormalities such as cytogenetic changes (balanced translocations, large inversions, etc.) or mitochondrial genome variants. These assays include array comparative genomic hybridization (CGH), traditional G-banded karyotyping, RNA analysis, long-range PCR, or mitochondrial DNA sequencing. Limited studies have demonstrated the use of such testing methods for hereditary hypophosphatemia.[82-84] Selecting the appropriate genetic test will depend on the patient's clinical presentation, prior genetic testing results, and family history.

Table 4. Summary of VUS Resolution Efforts

Variant	Classification change	Additional clinical information provided for reclassification	Reason for reclassification	Individuals impacted (*n*)
c.1109T>G (p.Met370Arg)	VUS > LP	Yes	Clinical information	5
c.1173+5G>A (Intronic)	VUS > LP	Yes	Segregation	2
c.1237G>C (p.Ala413Pro)	VUS > LP	Yes	Clinical information and segregation	5
c.1382C>G (p.Thr461Arg)	VUS > LP	Yes	Clinical information and segregation	4
c.1403A>C (p.Lys468Thr)	VUS > LP	Yes	Segregation (de novo)	1
c.1482+3A>C (Intronic)	VUS > P	Yes	Clinical information	4
c.1768+5G>A (Intronic)	VUS > P	No	Additional literature support	3
c.1936G>C (p.Asp646His)	VUS > LP	No	Found a patient with different variant at this codon	1
c.230G>A (p.Cys77Tyr)	VUS > LP	Yes	Clinical information	2
c.425G>T (p.Cys142Phe)	VUS > P	Yes	Segregation (de novo)	1
c.436+4A>G (Intronic)	VUS > P	No	Additional patients	5
c.499T>C (p.Trp167Arg)	VUS > LP	Yes	Segregation	5
c.503C>T (p.Pro168Leu)	VUS > LP	Yes	Clinical information	2
c.663G>A (Silent)	VUS > LP	Yes	Clinical information and segregation	4
Deletion (Exon 7)	VUS > LP	Yes	Clinical information	1
Gain (Exons 13–20)	VUS > LP	Yes	Segregation	3
c.1520T>C (p.Leu507Pro)	VUS (no change)	Yes	None available	NA
c.1702T>C (p.Ser568Pro)	VUS (no change)	Yes	None available	NA
c.1757T>C (p.Phe586Ser)	VUS (no change)	Yes	None available	NA
c.1850G>T (p.Cys617Phe)	VUS (no change)	Yes	None available	NA
c.1850G>T (p.Cys617Phe)	VUS (no change)	Yes	None available	NA
c.1973_1984dup (p.Arg658_Ile661dup)	VUS (no change)	Yes	None available	NA
c.2065G>C (p.Ala689Pro)	VUS (no change)	Yes	None available	NA
c.2147G>C (p.Arg716Thr)	VUS (no change)	Yes	None available	NA
c.2198G>T (p.Cys733Phe)	VUS (no change)	Yes	None available	NA
c.416A>G (p.Tyr139Cys)	VUS (no change)	Yes	None available	NA
c.482G>C (p.Arg161Pro)	VUS (no change)	Yes	None available	NA
c.934-16_934-12delinsCTAC CTAACTGAGAT (Intronic)	VUS (no change)	Yes	None available	NA
Gain (Exons 12–20)	VUS (no change)	Yes	None available	NA

LP = likely pathogenic; P = pathogenic; VUS = variant of uncertain significance.

The most commonly observed event among individuals with a positive *PHEX* molecular diagnosis was the combined presence of a gain of exons 13–15 and the 3'UTR c.*231A>G variant, seen in 65 probands and 23 family members tested either through the program at Invitae or outside of the program. Previous literature reports that identified the c.*231A>G variant as causative for XLH in six unrelated probands did not include copy number detection of *PHEX*.[85,86] Although the exact position of the gain of exons 13–15 in our cohort cannot be determined with the NGS assay used in this study, a large-scale analysis has found that the majority of subgenic gains are in tandem.[87] If this particular gain is in tandem, it would be predicted to create a frameshift because the duplicated exons are out of frame, suggesting that the causative variant may be the gain of exons 13–15 in these patients and not the c.*231A>G variant. Supporting this hypothesis, we did find one proband who had the gain without the c.*231A>G variant, suggesting that the duplication can contribute to disease on its own. However, further functional studies

to understand how one or both variants confer pathogenicity are needed. Our cohort, which to date represents the largest number of individuals with both variants ($n = 88$), including 51 individuals confirmed to harbor them in cis, supports the previous observation among eight patients that these two variants cosegregate in affected individuals.[81]

Recently, the c.*231A>G variant was reported to be associated with mild disease severity in a small cohort.[88] However, within our cohort, a wide spectrum of clinical features was reported for patients with both the gain of exons 13–15 and the c.*231A>G variant. Supporting our result, another large family with these variants, tested through the program, was recently reported to have symptoms with a wide spectrum of severity, including hearing loss.[89] These findings, together with the eligibility criteria for the sponsored testing program, do not suggest that these variants confer only mild disease.

For a subset of individuals included in this study, reported clinical features were used to characterize which features were associated with XLH and other disorders. The majority of individuals had a previous clinical diagnosis related to hypophosphatemia, with three-quarters having a confirmed molecular diagnosis through the sponsored program. Roughly 90% of patients who had a clinical diagnosis of XLH specifically, had a reportable variant (P/LP/VUS) identified in *PHEX*. Given the high probability of identifying a variant for patients with a confirmed XLH clinical diagnosis, at least some of the 10% of patients that did not have a detectable *PHEX* variant may harbor noncoding variants not detectable by current methods. We detected a small portion of individuals with molecular diagnosis for a gene other than *PHEX*, suggesting that another portion of the 10% of individuals without a *PHEX* variant may have a positive finding in another gene not included in the genetic test. A recent study demonstrated that RNA analysis is able to identify P/LP variants in deep intronic regions for a number of hereditary disorders across several clinical areas.[90] Patients with a confirmed XLH clinical diagnosis but no identifiable variant may also benefit from RNA analysis. The most common symptoms reported were lower limb deformities and short stature, though a confirmed family history of XLH; gait abnormalities; and bone pain, joint pain, and/or joint stiffness were also noted by clinicians. Notably, a small proportion of this cohort were *PHEX*-positive when no family or personal history was noted, suggesting the possibility of a *de novo* *PHEX* occurrence in those cases. Although parental testing was not available for the majority of the cohort to determine whether variants were *de novo*, previous studies have shown that the rate of sporadic cases are higher in female individuals for X-linked conditions, including XLH and other disorders.[91-93] This large dataset offers potential for learning more about the spectrum of disease across genotypes. Additional research into this cohort, in combination with prospective studies, is warranted to understand such relationships and to aid clinicians in managing patient care and tailoring treatments.

In addition to providing positive molecular diagnoses of XLH, the use of a multigene panel allowed for discernment between XLH and other forms of genetic hypophosphatemia that might be mistaken for XLH. In this study, several individuals with a suspected or confirmed clinical diagnosis of XLH received molecular diagnoses for other disorders, including autosomal recessive vitamin D–dependent rickets and hypophosphatasia. The identification of non-hypophosphatemia disorders such as HPP may have been due to their significant radiographic (but not biochemical) similarity to XLH.[94] Importantly, low serum phosphate was not an eligibility criterion, though clinicians had the opportunity to note it as optional information on the test requisition form. If low serum phosphate had been an eligibility requirement, the 31 individuals in our study who were found to have a molecular diagnosis in *ALPL* likely would have been ineligible. Molecular diagnoses in *ALPL* represented the majority of non-*PHEX*-positive results, most of which were in female individuals (as previously reported, which may also explain the higher proportion of female individuals in this group).[95-97] Equally important, 29.7% of individuals in the tested cohort received a negative result. Although 53.6% of these individuals had a hypophosphatemia-related clinical diagnosis, most had no family history of hypophosphatemia (82.8%). The most common reported clinical signs and symptoms were short stature (66.4%); muscle pain, weakness, or fatigue (44.5%); and bone, joint pain or joint stiffness (48.9%). The presence of these features without family history should not preclude a clinician from considering an XLH diagnosis. Although these data suggest that clinicians are adept at discriminating between genetic and nongenetic causes of hypophosphatemia, it is important for these patients to have follow-up for further evaluation. For example, some of these patients may harbor a variant in non-covered regions of the assay, such as a deep intronic pathogenic variant. Another group of these individuals may have a suspicious VUS in a gene that is consistent with their clinical presentation, warranting continued surveillance and reassessment of variant interpretation. In fact, 13 VUS in this cohort were reclassified as P/LP with additional information. Others may have nongenetic forms of osteomalacia (eg, tumor-induced osteomalacia),[98] and follow-up could help guide clinical management for this patient population.

Seven individuals who received a positive *PHEX* molecular diagnosis also had P/LP variants in other genes. Of note, two individuals had a single P/LP variant in *SLC34A3*, which is associated with autosomal recessive hereditary hypophosphatemic rickets with hypercalciuria (HRHH). Individuals who carry only one P/LP variant, though not a molecular diagnosis, have been shown to exhibit mild biochemical and renal findings.[69,79,80] Further, treatment for HRHH would be different from that of XLH, and an incorrect diagnosis could result in ineffective or potentially harmful interventions. In these cases, it is important to understand the impact of such complex genotypes on clinical presentation and indications (or contraindications) for various therapies.

Conventional treatment for XLH consists of oral phosphate and active vitamin D analog supplementation, which improves lower limb deformities, increases growth, and improves oral health.[99] Burosumab, approved by the U.S. Food and Drug Administration, has shown improved clinical outcomes related to normalization of serum phosphorus, healing of rickets, and improved growth.[100,101] Genetic testing, through sponsored programs such as this one, provides a means to confirm clinical diagnoses, which may be required by some third party payors for coverage of therapy.

A molecular diagnosis not only affects the individual who underwent testing but also has implications for family members. Once an affected individual receives a molecular diagnosis, clinicians should offer cascade testing of the proband's first-degree relatives, with subsequent positive results triggering additional cascade testing. Continuing to expand testing within families of an affected proband is critical, as the clinical presentation may vary across relatives who might not have been diagnosed or might have received an incorrect diagnosis. Cascade testing may result in an earlier diagnosis that consequently may lead

to clinical management that prevents or slows disease progression. In this cohort, 5.6% of probands with a molecular diagnosis in *PHEX* had family members pursue subsequent testing. Although such positive molecular diagnoses may have clear clinical utility for family members, additional awareness and education for clinicians should be pursued so that cascade testing is more consistently recommended when a positive molecular diagnosis is detected.

Through this sponsored testing program, 50 VUS in *PHEX* were detected in 53 individuals. In 26 cases, Invitae sought additional clinical information from the proband or family testing for segregation analysis in an effort to reclassify VUS as P/LP or (likely) benign. Sufficient information was available to reclassify 13 VUS as P/LP, ultimately impacting 39 individuals who had originally received a VUS result. There are many approaches to reclassifying VUS, each providing different types of evidence to aid in reinterpreting the variant.[77] In this study, seeking additional clinical information[102] or requesting family testing for segregation analysis[103] were leveraged for VUS resolution. The outcomes reported here show how effective these two methods are and highlight the importance of providing detailed clinical information to the testing laboratory for variant interpretation. Analyzing messenger RNA isoforms is another method, developed to aid in VUS reclassification when a variant is suspected to disrupt a splice site. RNA analysis of an individual's sample may inform whether the variant alters gene expression, though it would be required for the gene of interest to be expressed in the sample provided to the testing laboratory.[104,105] Functional studies in model systems and cell lines that investigate protein levels, interactions, reactivity, or other functions in relation to the VUS may also provide evidence for VUS reclassification, though these studies have their own limitations.[106] As a VUS does not have enough evidence to suggest that the variant is pathogenic (or benign), this result may leave clinicians and patients in a state of uncertainty, possibly excluding patients from medical treatments that require a positive molecular diagnosis. Thus, it is critical for genetic testing laboratories to continually revisit VUS and utilize all available information for reclassification.

An important benefit to a sponsored testing program is increased access to testing and care. One critical element of access is the recent decreases in costs associated with genetic testing and the advances in technology that allow for more detailed investigation of the genome in a single assay. Genetic testing has been recommended as a means to confirm a clinical diagnosis of XLH and enabling screening of at-risk relatives, though it is not required for a diagnosis.[107] In spite of this, a positive molecular genetic test result is often an eligibility requirement for enrolling in clinical trials or receiving targeted therapies. Collaborative efforts among clinicians, pharmaceutical companies, and genetic testing laboratories can aid in increasing access to genetic testing, trials, or treatments that may improve clinical outcomes and in robustly characterizing the genetic spectrum of disease. As more collaborations occur to provide genetic testing results, the full benefits, and possible disadvantages, will become increasingly apparent.

In considering the results from this study, analysis of the study design reveals that interpretation of clinical features associated with XLH may be limited. As part of the eligibility criteria, clinicians were required to report if an individual had a confirmed or suspected XLH diagnosis. Further, clinical signs and symptoms were reported only for individuals with suspected diagnoses (ie, those displaying at least two signs or symptoms). Thus, in addition to a limited number of individuals with reported clinical information, there may have been variability in reporting from clinician to clinician. In addition, multigene panels query a specific set of genes and do not allow for identifying causative variants in other genes, including novel genes not currently linked to genetic hypophosphatemia. However, the panel utilized in this cohort was designed to capture the most common genes associated with hypophosphatemia and since the end of this study period and data cut, four additional genes (*CTNS*, *GNAS*, *OCRL*, and *SLC34A1*) have been added to the panel.

Taken together, the results from this study show the benefits of sponsored testing programs for identifying and/or confirming diagnoses for rare diseases, as evidenced by the high diagnostic yield and novel insights into the genetic landscape contributing to XLH. Additionally, we were able to leverage the results of the program to identify novel variants and understand more about phenotypic variability and provider ordering preferences. As more individuals undergo testing, future analysis of the sponsored Hypophosphatemia program will continue to be an invaluable resource in understanding *PHEX*-related hypophosphatemia.

Disclosures

Eric T. Rush and Norma E. Guerra have received consulting and speaking fees from Ultragenyx Pharmaceutical Inc. Kathryn Dahir has received consulting and speaking fees from Ultragenyx Pharmaceutical Inc. and is an X-Linked Hypophosphatemia Disease Monitoring Program (XLH-DMP) investigator. Britt Johnson, Swaroop Aradhya, Daniel Beltran, Sara L. Bristow, Ana Morales, and Rebecca Truty are shareholders and employees of Invitae. Scott Eisenbeis, Stan Krolczyk, Nicole Miller, Prameela Ramesan, and Soodabeh Sarafrazi are employees of Ultragenyx Pharmaceutical Inc. SE, SK, NM, and PR, are shareholders of Ultragenyx Pharmaceuticals, Inc.

Acknowledgments

We thank Karen Ouyang, PhD, Sarah Poll, PhD, Dianalee McKnight, PhD, and Kerry Aradhya, employees of Invitae, for assistance with data analysis or critical review of the manuscript. This study was sponsored and funded by Ultragenyx Pharmaceutical Inc in partnership with Kyowa Kirin International plc.

Authors' roles: Substantial contributions to study conception and design: BJ, SA, SE, SK, NM, PR; Substantial contributions of data: ETR, NEG, KD; Substantial contributions to analysis and interpretation of data: ETR, BJ, SA, DB, SLB, SE, NEG, SK, NM, AM, PR, SS, RT, KD; Drafting the article or revising it critically for important intellectual content: ETR, BJ, SA, DB, SLB, SE, NEG, SK, NM, AM, PR, SS, RT, KD; Final approval of the article to be published: ETR, BJ, SA, DB, SLB, SE, NEG, SK, NM, AM, PR, SS, RT, KD; Authors who accessed and verified the data: BJ, SLB, SS, RT.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request. All variants reported in this article have been submitted to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/).

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