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Clinical Research Article

ALPL Genotypes in Patients With Atypical Femur Fractures or Other Biochemical and Clinical Signs of Hypophosphatasia

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Abstract

Context: Hypophosphatasia (HPP) is a rare metabolic disorder caused by deficiency of alkaline phosphatase (ALP) enzyme activity, leading to defective mineralization, due to pathogenic variants of the *ALPL* gene, encoding the tissue nonspecific alkaline phosphatase (TNSALP) enzyme. Inheritance can be autosomal recessive or autosomal dominant. An abnormal *ALPL* genetic test enables accurate diagnosis, avoiding the administration of contraindicated antiresorptive drugs that, in patients with HPP, substantially increase the risk of atypical femur fractures (AFFs) and worsen the fracture healing process that is usually already compromised in these patients.

Objective: Performing *ALPL* genetic testing to identify rare variants in suspected adult patients with HPP. Comparing frequencies of *ALPL* common variants in individuals with biochemical and/or clinical signs suggestive of adult HPP and non-HPP controls, and among different clinical subgroups of patients with a clinical suspicion of adult HPP.

Methods: Patients with suspected adult HPP were retrospectively selected for the genetic testing of the *ALPL* gene. Patients included were from 3 main European Bone Units (Florence, Naples, and Geneva); 106 patients with biochemical and/or clinical signs suggestive of a mild form of HPP were included.

Results: Genetic testing led to the identification of a heterozygote rare variant in 2.8% of cases who were initially referred as suspected osteoporosis. The analysis of frequencies of *ALPL* common variants showed a high prevalence (30.8%) of homozygosity in subjects who developed an AFF, in association with normal serum total ALP activity.

Conclusion: The results suggest homozygosity of common *ALPL* variants as a possible genetic mark of risk for these fractures.

Key Words: hypophosphatasia, tissue nonspecific alkaline phosphatase (TNSALP), *ALPL* gene, rare variants, common variants, atypical femur fracture (AFFs)

Abbreviations: AFF, atypical femur fracture; ALP, alkaline phosphatase; BMD, bone mineral density; HPP, hypophosphatasia; TNSALP, tissue nonspecific alkaline phosphatase.

Hypophosphatasia (HPP) is a rare metabolic disorder caused by deficiency of alkaline phosphatase (ALP) enzyme activity, leading to defective bone and tooth mineralization. Six categories have been described, following a classification based on the age of diagnosis, which is associated with relative severity: perinatal lethal, prenatal benign, infantile, childhood, adult, and odonto-HPP (1). Generally, the younger the age of onset, the more severe the clinical phenotype is, ranging from a perinatal lethal form, with virtually no skeletal mineralization, to mild forms with late adult onset (1, 2). While during infancy and childhood the diagnosis of HPP can readily be made, based on specific clinical, radiographic, and basic laboratory findings, a clear diagnosis of adult HPP is more difficult since adults commonly present a varying spectrum of nonspecific manifestations. Many adults with HPP report the occurrence of some symptoms during their childhood, but the diagnosis is commonly not made until later in life (1, 2). Atypical femur fractures (AFFs) and stress fractures are 2 clinical hallmarks of adult HPP (1, 2). AFFs are fragility fractures affecting the subtrochanteric or diaphyseal area of the femur with a transverse morphology, originating at the lateral cortex, occurring in patients with HPP mainly after prolonged treatment with antiresorptive drugs. Stress fractures occur as a consequence of physical stress on the metatarsal bones or tibia, and they can be recurrent, multiple, and commonly slow to heal.

Low serum activity of unfractionated ALP is a biochemical hallmark of HPP, that, however, is not diagnostic by itself, as

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it can be associated with other disorders, or with prior/concomitant treatment with antiresorptive drugs, glucocorticoids, or chemotherapy (3). In the presence of low serum activity of total ALP, additional biochemical tests (ie, serum and/or urine measurements of pyridoxal 5′-phosphate [vitamin B6, PLP] and phosphoethanolamine) (4), and the recognition of HPP-compatible clinical signs and radiological findings are fundamental for the appropriate diagnosis of HPP. The mildest forms of the disease are commonly under- and/or misdiagnosed because of their heterogeneity of signs and symptoms, which can overlap with those caused by other metabolic bone diseases. Differentiation between adult HPP and osteoporosis is important since in patients with HPP the administration of antiresorptive agents could further impair bone mineralization and increase the risk of AFFs (5, 6). Genetic testing can confirm the diagnosis and allows appropriate genetic counseling.

HPP is caused by loss-of-function rare variants of the *ALPL* gene (MIM 171760), encoding the tissue nonspecific alkaline phosphatase (TNSALP) enzyme. The *ALPL* gene is localized on chromosome 1p36.1-34 and consists of 12 exons distributed over 70 kb (7).

Up to January 2021, the Tissue Nonspecific Alkaline Phosphatase Gene Mutations Database was hosted at the University of Versailles website (http://alplmutationdatabase.hypophosphatasie.com), reporting 411 different mutations of the *ALPL* gene. Currently, the ALPL Gene Variant Database is hosted at the Johannes Kepler University Linz website (https://alplmutationdatabase.jku.at/), and it includes more than 400 mutations (rare variants presenting a global minor allele frequency less than 1% in the general population), classified into benign, likely benign, unknown significance, likely pathogenic, and pathogenic.

Several pathogenic variants of the *ALPL* gene, differently affecting the degree of activity of the enzyme, have been associated with different mechanisms of disease and potentially with degrees of clinical severity. Severe forms of HPP typically have an autosomal recessive inheritance and are caused by homozygous or compound heterozygous loss-of-function variants (8, 9). The mildest forms are typically inherited in an autosomal dominant fashion, caused by heterozygous *ALPL* variants (8, 9). The catalytic activity of TNSALP enzyme requires homodimeric conformation. Functional studies have demonstrated that some heterozygous *ALPL* variants may exert a dominant negative effect, in which the activity of the wild-type monomer of TNSALP is suppressed by the variant monomer (10–12). Recently, Mornet et al. (13) classified *ALPL* variants into 4 functional types: (1) normal (N); (2) moderate (m); (3) severe with no dominant negative effect (s); and (4) severe with dominant negative effect (Sd). According to this classification of *ALPL* variants and resulting genotypes, the authors classified HPP disease into 3 main clinical groups: (1) severe forms (primarily perinatal and infantile) inherited as autosomal recessive tract with a prevalence of 1:300 000 and associated with s/s, Sd/s, Sd/Sd, or m/m alleles; (2) moderate forms (infantile, childhood, odonto-HPP, and typical adult HPP) showing both autosomal dominant or autosomal recessive patterns of inheritance with a prevalence of about 1:2430 and associated with Sd/m, s/m or Sd/N alleles; and (3) mild forms (adult presentation with nonspecific clinical signs) with an autosomal dominant inheritance, presumably due to *ALPL* haploinsufficiency, and associated with s/N or m/N alleles. Adults with s/N or m/N genotypes may present borderline serum levels of ALP and are less severely affected than adults with Sd/N alleles (13).

In addition to its numerous described pathogenic rare variants, the *ALPL* gene is also characterized by a high heterogeneity of common variants. Sixteen common variants, having a global minor allele frequency higher than 1% in the general population, were reported, as polymorphisms, in the Tissue Nonspecific Alkaline Phosphatase Gene Mutations Database up to January 2021: 3 missense variants, 5 silent variants, and 8 intronic variants. A table of these common variants is not currently updated on the ALPL Gene Variant Database. Some studies have suggested a possible functional effect of some of these common variants on the TNSALP activity and on bone mineralization. The 2 major allele frequencies of 2 single nucleotide polymorphisms, c.787 T>C (p.Y263H) in exon 7 and c.876 A>G (p.P292P) in exon 9, and their resulting haplotype, have been associated with a higher bone mineral density (BMD) of the radial bone in Japanese post-menopausal women (14). The presence of the histidine, instead of tyrosine, at codon 263, increased the kinetic affinity of TNSALP for its substrates and may have a regulatory effect on bone mineralization and BMD (14, 15). Ermakov et al. (16) tested the association of 42 single nucleotide polymorphisms in the *ALPL* locus, and 39 resulting haplotypes, with bone strength–related traits, in 1253 Caucasian individuals. The study showed that *ALPL* polymorphisms located in the region of the gene corresponding to exons 7-9 were associated with both bone size at various skeletal sites and hand BMD, and they may presumably have functional effects on TNSALP activity and bone metabolism.

Considering this, the presence of specific common variants of the *ALPL* gene could recapitulate a mild HPP phenotype, even in absence of *ALPL* known pathogenic variants. In a recent study, we showed that the presence of 3 or more of these *ALPL* common variants was associated with significantly lower serum levels of total ALP activity and with a higher prevalence of metatarsal fractures, supporting the hypothesis that *ALPL* common variants may resemble a mild HPP phenotype, even in individuals negative for *ALPL* pathogenic variants (17).

In the present study, we performed genetic testing of the coding region and intron–exon junctions of the *ALPL* gene, comparing the frequencies of *ALPL* common variants between individuals with biochemical and/or clinical signs supportive of adult HPP and non-HPP controls, and among different clinical subgroups of these patients with HPP, to identify if the presence of 1 or more of these common variants could represent an informative marker for HPP risk and/or the occurrence of specific HPP-related phenotypical manifestations, such as AFFs.

Patients and Methods

Patients

This study was approved by the Review Board of the Area Vasta Centro, Regione Toscana at the Azienda Ospedaliera-Universitaria Careggi (ref. CEAVC 10601_oss). Clinical data and blood samples were collected and analyzed anonymously. Each enrolled subject was identified by a unique alphanumeric code during the entire study.

The study included 89 patients with biochemical and clinical signs ascribable to suspected adult HPP (including 9 patients with AFF), retrospectively selected from ambulatory cases referred to the Hospital Outpatient Clinic of the Metabolic Bone Disease Unit of the Azienda Ospedaliero-Universitaria Careggi from 2015 to 2020, and 17 patients with AFF recruited from 2 Clinical Bone Units in Naples (4 cases) and Geneva (13 cases).

Data on fragility fractures, dental disease, chondrocalcinosis, nephrocalcinosis, poorly healing fractures, musculoskeletal pain, serum levels of total ALP, bone ALP and vitamin B6, serum markers of bone and mineral metabolism (ie, serum calcium, phosphate, intact parathyroid hormone, and 25(OH)-vitamin D, and urinary calcium and phosphate), and dual-energy X-ray absorptiometry parameters were retrospectively retrieved from medical records, when available.

The *ALPL* genetic testing was performed in the 106 patients (89 women and 17 men; mean age 51.3 ± 18.6 years, range 14-90 years) with biochemical and/or clinical signs of adult HPP (patients with HPP), and in 52 individuals (25 women and 27 men; mean age 44.7 ± 18.9 years, range 14-76 years) with serum values of total ALP activity and vitamin B6 within the normal ranges and no signs or symptoms suggestive of HPP (non-HPP controls).

Out of the 106 HPP cases, 65 patients (52 women and 13 men, mean age 46.0 ± 16.5 years, range 14-82 years) had low serum values of total ALP activity, while 41 (37 women and 4 men, mean age 60.5 ± 18.6 years, range 18-90 years) presented normal values of serum total ALP activity. These 106 patients with HPP were classified in subgroups, based on their adult HPP-related clinical phenotypes, as it follows:

1. 26 patients (23 women and 3 men; mean age 67.5 ± 17.6 years, range 18-90 years) with AFFs (2 with low serum values of total ALP activity and 24 with normal or borderline serum values of total ALP activity);
2. 19 patients (16 women and 3 men; mean age 45.1 ± 12.7 years, range 26-71 years) with low serum values of total ALP activity and no fragility fractures;
3. 44 patients (35 women and 9 men; mean age 45.1 ± 17.2 years, range 14-82 years) with low serum values of total ALP activity and at least 1 fragility fracture, other than AFF;
4. 11 patients (9 women and 2 men; mean age 52.8 ± 15.0 years, range 18-76 years) with serum values of total ALP activity within the normal range, but stress metatarsal fracture and/or other clinical signs of adult HPP (ie, poorly healing fracture, premature tooth loss and/or periodontal disease, musculoskeletal pain, nephrocalcinosis, chondrocalcinosis);
5. 6 women (mean age 53.5 ± 20.3 years, range 28-87 years) with serum values of total ALP activity within the normal range, but elevated serum levels of vitamin B6.

ALPL genetic analysis

Genetic screening of the *ALPL* gene was performed by Sanger sequencing of coding region (exons 2-12) and intron–exon junctions (covering at least 100 intronic bp) of the gene. Sequencing was performed with BigDye Terminator cycle sequencing reagents (Applied Biosystems, Carlsbad, CA, USA) and analyzed on ABI Prism 3100 Genetic Analyzer. Sequences were compared with the human *ALPL* wild-type sequence (transcript reference NM_000478.6; genomic reference NG_008940.1). The standardized nomenclature (protein reference NP_000469.3) was used to report both rare and common variants, counting from the first codon (ATG initiation), according to the recommendations of the Human Genome Variation Society.

The Polymorphism Phenotyping 2 tool (PolyPhen-2 v2.2.2r406; freely available at http://genetics.bwh.harvard.edu/pph2/), predicting the possible impact of an amino acid substitution on the structure and function of a human protein, was used in silico to assess the possible functional pathogenicity of the identified missense rare variants on the TNSALP protein activity.

Statistical Analyses

Differences in frequencies of common variants in the *ALPL* gene, along with phase and state of variants, were compared between HPP cases and non-HPP controls, as well as among different clinical subgroups of patients with HPP, by using the Fisher's exact test. Statistical significance was considered with $P < .01$.

Results

Rare Variants of the *ALPL* Gene

Genetic testing of the *ALPL* gene in our 106 suspected cases of adult HPP led to the identification of a heterozygous rare variant in 3 patients (2.8%). One variant was identified in the subgroup of patients with AFF (1/26; 3.8%), 1 in the subgroup of patients with low serum value of total ALP activity and no fragility fracture (1/19; 5.3%), and 1 in the subgroup of patients with low serum value of total ALP activity with fragility fractures other than AFF (1/44; 2.3%).

The *ALPL* variant identified in a patient with AFF was the heterozygote c.818 C>T substitution in exon 8, resulting in a nonconservative amino acid replacement at codon 273 of the TNSALP protein (p.Thr273Met; p.T273M), which is not reported either in the ALPL Gene Variant Database or in the Human Gene Mutation Database (http://www.hgmd.cf.ac.uk/ac/all.php). The Clinvar website indicates this variation with conflicting interpretations of its pathogenicity (https://www.ncbi.nlm.nih.gov/clinvar/variation/288247/). Three entries classified it as benign, while 2 others classified it as a variant of uncertain clinical significance. The 273 threonine to methionine change occurs at a position that is conserved in mammals, and it is likely to affect the protein secondary structure, since these 2 amino acids differ in polarity, charge, size, and other properties. The PolyPhen-2 analysis predicted the threonine to methionine substitution at codon 273 as benign on the protein structure/function, with a score of 0.287 (sensitivity: 0.91; specificity: 0.89).

The *ALPL* variant found in a patient with low serum value of total ALP activity and no fragility fracture was the heterozygous c.512 A>G substitution in exon 6 that results in a nonconservative amino acid substitution at codon 171 of the TNSALP protein (p.His171Arg; p.H171R). This variant is reported in the ALPL Gene Variant Database as associated both with infantile (18) and adult (19, 20) forms of HPP. There is no Clinvar entry for this variant. The PolyPhen-2 analysis predicted the histidine to arginine substitution at codon 171

as probably damaging on the protein structure/function, with a score of 0.999 (sensitivity: 0.14; specificity: 0.99).

The *ALPL* variant identified in a patient with low serum value of total ALP activity and fragility fractures, other than AFF, was the heterozygote c.571 G>A substitution in exon 6, resulting in a nonconservative substitution at codon 191 of the TNSALP protein (p.Glu191Lys; E191K). This variant is reported in the ALPL Gene Variant Database as associated with prenatal benign (18), infantile (13, 18, 20-23), childhood (18, 22, 24, 25), adult (18), and odonto- (18) HPP forms. On the Clinvar website (https://www.ncbi.nlm.nih.gov/clinvar/variation/13670), this mutation is reported as pathogenic by several entries and likely pathogenic by 2 entries. The PolyPhen-2 analysis predicted the glutamic acid to lysine substitution at codon 191 as probably damaging on the protein structure/function, with a score of 0.973 (sensitivity: 0.77; specificity: 0.96).

Common Variants of the *ALPL* Gene

Sequencing of the *ALPL* coding region and intron–exon junctions in our series of patients identified the presence of an additional common variant in intron 8 (c.863-46 G>A), not previously reported in the Tissue Nonspecific Alkaline Phosphatase Gene Mutation Database, which showed an incomplete linkage disequilibrium with the other 2 common variants lying nearby in intron 8 (c.863-7 T>C and c.863-12 C>G).

The presence of 2 out of the 17 analyzed *ALPL* common variants were not found in all the 106 patients with HPP and the 52 non-HPP controls (c.510 C>T in exon 6 [p.N170N] and c.1266 G>A in exon 11 [p.K422K]).

No significant differences were found in frequencies of all the other 15 common variants between patients with HPP and non-HPP controls.

The frequency of the c.472 + 12delG variant was significantly higher ($P = .0001$) in individuals with normal serum values of total ALP activity (19/41; 46.3%), with respect to those with a reduced enzymatic activity (5/65; 7.7%). Conversely, all the other 14 common variants showed no significant difference in their frequencies between these 2 subgroups of patients.

None of the 15 common variants showed a significantly different distribution among different clinical subgroups of patients with HPP.

The presence of homozygosity, in at least 1 HPP patient or non-HPP control, was found in 12 of the *ALPL* common variants. Frequencies of homozygous *ALPL* common variants in patients with HPP and non-HPP controls are reported in Table 1. No significant difference was found in frequencies of either single homozygous variants or global variant homozygosity between patients with HPP and non-HPP controls. The subgroup of 26 patients with AFF showed a high frequency of total variant homozygosity (8/26; 30.8%) that was significantly higher ($P = .0012$) than that of the other 80 patients with HPP (4/80; 5.0%) and higher than that of the subgroup of patients with HPP with low serum values of ALP activity (3/63, 4.8%) ($P = .0019$). No other significant difference was found either in the frequencies of single homozygous variants or in the total variant homozygosity among all the other different clinical subgroups of patients with HPP.

Discussion

Long-term treatment with the commonly used antiresorptive drugs has been shown to favor the occurrence of AFFs (26), a risk that is substantially higher in HPP adults misdiagnosed for osteoporosis and treated with bisphosphonates or denosumab (5, 6), or in HPP children following bisphosphonate therapy for an incorrect diagnosis of osteogenesis imperfecta. Appropriate diagnosis of HPP to distinguish it from antiresorptive responsive bone disorders is of great importance to avoid administration of contraindicated drugs that can increase the occurrence of AFFs and further slow fracture healing.

Clearly, abnormal *ALPL* genetic testing is decisive in supporting a diagnosis of HPP, especially in the prenatal context where clinical, imaging, and biochemical screenings are limited, and in adults presenting with nonspecific symptoms. Unfortunately, a significant percentage of patients with mild to moderate forms of adult HPP are negative for pathogenic *ALPL* variants. In these cases, the presence of certain common variant(s) in the *ALPL* gene could influence the activity of the TNSALP enzyme and contribute to the development of a mild HPP phenotype. It is also possible that some of these common variants are additive and may explain disease variation.

In our series of patients with clinical suspicion of an adult form of HPP, genetic testing of the *ALPL* gene identified a rare heterozygote variant in 2.8% of cases. All 3 patients were initially referred to clinical centers for suspected osteoporosis. The identification of an *ALPL* variant enabled a modification of the therapeutic approach and follow-up, according to the results of the genetic test.

The patient with an AFF and an *ALPL* rare variant had been receiving ibandronate therapy for 20 years before an AFF fracture occurred at the age of 90 years. No data on other clinical signs or symptoms attributable to HPP and serum values of vitamin B6 were available in the medical records. Two serum measurements of total ALP activity, performed the same year as the AFF, showed inconsistent results, 1 being borderline and another within the normal range. The same year, a dual-energy X-ray absorptiometry analysis showed osteopenia at the lumbar spine (L1-L3; T-score –1.5), but it was not performed at femur sites. The heterozygous variant identified in this patient (p.T273M) is not recorded in the ALPL Gene Variant Database and its effect trends are benign. In silico analysis indicated it as benign on the TNSALP protein structure/function. Nielson et al. (27) associated the presence of this heterozygous variant with individuals having serum ALP activity below the clinical lower limit of normal and presenting a low BMD, in comparison with individuals with serum ALP levels near the median of the population distribution. Interestingly, this patient is carrier also of 8 different *ALPL* common variants, all in homozygote status, and located within the region of exons 7-9 which has previously been identified as possibly involved in TNSALP activity (16). Thus, we speculate that the p.T273M variant could be a low penetrance mutation, exerting a role on the development of a moderate HPP phenotype presumably in an additional fashion with other *ALPL* common variants.

Both the other 2 patients bearing an *ALPL* rare variant presented with reduced serum values of total ALP activity and bone ALP and increased serum levels of vitamin B6. One of them had manifested diffuse musculoskeletal pain since infancy, prevalently at the spine and myalgia of the legs and

Table 1. Frequencies of monozygous *ALPL* common variants in HPP patients and non-HPP controls

Patient clinical group	c.472 + 12delG, intron 5	c.793-31C>T, intron 7	c.787 T>C (p.Y263H), exon 7	c.862 + 20 G>T, intron 8	c.862 + 51 G>A, intron 8	c.862 + 58 C>T, intron 8	c.863-7 T>C, intron 8	c.863-12 C>G, intron 8	c.863-46 G>A, intron 8	c.876 A>G (p.P292P), exon 9	c.1542 G>T (p.A514A), exon 12	c.1565 T>C (p.V522A), exon 12	Total variant homozygosity
Patients with HPP													
Total patients with biochemical and/or clinical signs of adult HPP (n = 106)	5/106 (4.7%)	4/106 (3.8%)	1/106 (0.9%)	1/106 (0.9%)	1/106 (0.9%)	2/106 (1.9%)	1/106 (0.9%)	1/106 (0.9%)	1/106 (0.9%)	1/106 (0.9%)	1/106 (0.9%)	0/106 (0%)	12/106 (11.3%)
Total patients with HPP with low serum values of ALP activity (n = 65)	1/65 (1.5%)	2/65 (3.1%)	0/65 (0%)	0/65 (0%)	0/65 (0%)	0/65 (0%)	0/65 (0%)	0/65 (0%)	0/65 (0%)	0/65 (0%)	0/65 (0%)	0/65 (0%)	3/65 (4.6%)
Total patients with HPP with normal serum values of ALP activity (n = 41)	4/41 (9.8%)	2/41 (4.9%)	1/41 (2.4%)	1/41 (2.4%)	1/41 (2.4%)	2/41 (4.9%)	1/41 (2.4%)	1/41 (2.4%)	1/41 (2.4%)	1/41 (2.4%)	1/41 (2.4%)	0/41 (0%)	9/41 (22.0%)
Patients with atypical femur fracture (n = 26)	3/26 (11.5%)	2/26 (7.7%)	1/26 (3.8%)	1/26 (3.8%)	1/26 (3.8%)	2/26 (7.7%)	1/26 (3.8%)	1/26 (3.8%)	1/26 (3.8%)	1/26 (3.8%)	1/26 (3.8%)	0/26 (0%)	8/26 (30.8%)
Patients with low serum values of ALP activity and no fragility fracture (n = 19)	1/19 (5.3%)	1/19 (5.3%)	0/19 (0%)	0/19 (0%)	0/19 (0%)	0/19 (0%)	0/19 (0%)	0/19 (0%)	0/19 (0%)	0/19 (0%)	0/19 (0%)	0/19 (0%)	2/19 (10.5%)
Patients with low serum value of ALP activity and fragility fracture, other than atypical femur fracture (n = 44)	0/44 (0%)	1/44 (2.3%)	0/44 (0%)	0/44 (0%)	0/44 (0%)	0/44 (0%)	0/44 (0%)	0/44 (0%)	0/44 (0%)	0/44 (0%)	0/44 (0%)	0/44 (0%)	1/44 (2.3%)
Patients with normal serum values of ALP activity but elevated serum levels of vitamin B6 (n = 6)	1/6 (16.7%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	0/6 (0%)	1/6 (16.7%)

Table 1. Continued

Patient clinical group	c.472 + 12delG, intron 5	c.793-31C>T, intron 7	c.787 T>C (p.Y263H), exon 7	c.862 + 20 G>T, intron 8	c.862 + 51 G>A, intron 8	c.862 + 58 C>T, intron 8	c.863-7 T>C, intron 8	c.863-12 C>G, intron 8	c.863-46 G>A, intron 8	c.876 A>G (p.P292P), exon 9	c.1542 G>T (p.A514A), exon 12	c.1565 T>C (p.V522A), exon 12	Total variant homozygosity
Patients with normal serum values of ALP activity but metatarsal fracture and/or other clinical signs of adult HPP (n = 11)	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)	0/11 (0%)
Non-HPP controls													
Non-HPP controls (n = 52)	1/52 (1.9%)	1/52 (1.9%)	1/52 (1.9%)	1/52 (1.9%)	1/52 (1.9%)	4/52 (7.7%)	1/52 (1.9%)	1/52 (1.9%)	1/52 (1.9%)	1/52 (1.9%)	0/52 (0%)	1/52 (1.9%)	7/52 (13.5%)

feet, spontaneous loss of 2 teeth at the age of 30, and multiple spontaneous vertebral fractures before the age of 60. The genetic diagnosis of HPP at the age of 64 years led to alternate clinical management, including medical therapy with asfotase alfa, which resulted in a reduction of musculoskeletal pain after 6 months of treatment, a significant increase in ALP serum levels, and a substantial decrease in vitamin B6 levels. The second patient showed neither fragility fractures nor other clinical signs and symptoms ascribable to HPP, except for diffuse musculoskeletal pain at the spine, wrists, and ankles. A genetic test confirmed the diagnosis of HPP at the age of 66 years, preventing this patient from receiving further medical therapy with bisphosphonates after the diagnosis.

The *ALPL* gene presents a significant molecular heterogeneity both for HPP-causative pathogenic variants and for common variants. Sixteen such common *ALPL* variants are reported in the Tissue Nonspecific Alkaline Phosphatase Gene Mutations Database. In this study, we identified an additional polymorphic variant in intron 8 (c.863-46 G>A), which appeared to be in incomplete linkage disequilibrium with the other 2 polymorphic variants in the 5′ extremity of intron 8.

In our series of patients, the frequency of the c.472 + 12delG common variant in intron 5 was significantly higher in individuals with normal serum values of total ALP activity with respect to those with low total ALP serum levels. This intronic variant is reported as benign in Clinvar. In silico analyses predict that the G deletion, 12 bp downstream of exon 5, does not affect correct splicing, suggesting this variant does not directly affect TNSALP structure and activity, even if specific functional studies have not been performed. Therefore, the positive association of this intronic variant with serum values of ALP activity in our patients could be accidental, due to the few numbers of variant observations in the subgroup of patients with normal serum ALP (4/65), which could have reduced the strength of the statistical analysis, and must be replicated in larger populations.

Mornet et al. (13) found that the homozygosity of *ALPL* pathogenic mutations was a determinant of aggravating severity of HPP. In their study population, the rate of homozygosity was extremely high (65% for Sd/Sd and s/s genotypes) in the lethal perinatal form, and patients with the HPP infantile form bearing an m variant showed 100% homozygosity for the allele. Interestingly, in our series of patients, a high prevalence of homozygosity for *ALPL* common variants was found in the subgroup of patients with AFF (30.8%), significantly higher than the subgroup of all the other 80 patients with HPP and the subgroup of the other patients with HPP with low levels of ALP, while no significance was found with respect to the other patients with HPP showing normal serum levels of ALP or the non-HPP controls. Since a great majority of our patients with AFF showed normal or borderline serum values of total ALP activity (24/26; 92.3%), the occurrence of these fractures appears to be independent of TNSALP activity. As speculated by Mornet et al. (13) for hypomorphic alleles of *ALPL* rare variants, we could also assume for homozygous common variants a negative interaction between the encoded variant TNSALP protein and another actor of bone mineralization, like the collagen matrix. Alternatively, AFF occurrence may be the result of other genetic influences acting on bone remodeling and mineralization processes. Common variants in genes involved in the regulation of bone mineralization, even if not directly responsible on their own for full clinical development of HPP, could additively impact, together with

ALPL common variants, mineralization in mild forms of HPP, even in the presence of normal and/or borderline total ALP serum activity. However, a recent study of Kharazmi et al. (28) failed to find any significant association between genetic variants and the occurrence of bisphosphonate-associated AFFs, both after a genome-wide association study including human common genetic variants and a target analysis of 29 candidate genes that had previously been associated with AFF development (29).

In our patients, the presence of homozygosity for 1 or more *ALPL* common variants appears to be a marker associated with the occurrence of AFFs in almost 1 of 3 patients, suggesting that this genotype feature could have a possible pharmacogenetic application in predicting individuals at higher risk of developing these fractures as a consequence of antiresorptive therapy with bisphosphonates and denosumab. Studies on a larger population are necessary to confirm our data.

Conclusions

In the adult expression of HPP signs and symptoms are milder than in earlier onset of the disease. Diagnosis in adults can be difficult. Pathogenic variants of the *ALPL* gene are not always found even in patients with low serum ALP values, high circulating levels of PLP, and a skeletal phenotype typical of HPP. Interestingly, the prescription of asfotase alfa does not require a positive genetic test of *ALPL* mutations. The present study evidenced as homozygosity for common variants of the *ALPL* gene segregated with AFF, a skeletal complication of the disease. These findings open the possibility for clinicians of evaluating patients with low serum ALP genetically negative for *ALPL* gene mutations, as well as AFF patients, for common variants of the gene. Such information could improve the management of these difficult cases.

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Disclosure Summary

All the authors declare that they have no competing interests, associated with this manuscript.

Data Availability

The datasets analyzed in the current study are not publicly available, but they are available from the corresponding author upon reasonable request.

References

1. Mornet E. Hypophosphatasia. *Orphanet J Rare Dis*. 2007;2:40.
2. Whyte PM. Hypophosphatasia: an overview for 2017. *Bone*. 2017;102:15-25.
3. Macfarlane JD, Souverijn JH, Breedveld FC. Clinical significance of a low serum alkaline phosphatase. *Netherlands J Med*. 1992;40(1-2):9-14.
4. Whyte MP. Hypophosphatasia: nature's window on alkaline phosphatase function in humans. In: Bilezikian JP, Raisz LG, Martin TJ, ed. *Principles of Bone Biology*. 3rd ed. Academic Press; 2008:1573–1598.
5. Sutton RAL, Mumm S, Coburn SP, Ericson KL, Whyte MP. Atypical femoral fractures during bisphosphonate exposure in adult hypophosphatasia. *J Bone Miner Res*. 2012;27(5):987-994.
6. Tsuchie H, Miyakoshi N, Kasukawa Y, *et al*. Evaluation of the nature and etiologies of risk factors for diaphyseal atypical femoral fractures. *Med Princ Pract*. 2021;30(5):430-436.
7. Su N, Zhu M, Cheng X, Xu K, Kocijan R, Zhang H. Six ALPL gene variants in five children with hypophosphatasia. *Ann Transl Med* 2021;9(10):888.
8. Mornet E. Molecular genetics of hypophosphatasia and phenotype-genotype correlations. *Subcell Biochem*. 2015;76:25-43.
9. Mornet E. Genetics of hypophosphatasia. *Arch Pediatr*. 2017;24(5S2):5S51-5S56.
10. Lia-Baldini AS, Brun-Heath I, Carrion C, *et al*. A new mechanism of dominance in hypophosphatasia: the mutated protein can disturb the cell localization of the wild-type protein. *Hum Genet*. 2008;123(4):429-432.
11. Lia-Baldini AS, Muller F, Taillandier A, *et al*. A molecular approach to dominance in hypophosphatasia. *Hum Genet*. 2001;109(1):99-108.
12. Muller HL, Yamazaki M, Michigami T, *et al*. Asp361Val Mutant of alkaline phosphatase found in patients with dominantly inherited hypophosphatasia inhibits the activity of the wild-type enzyme. *J Clin Endocrinol Metab*. 2000;85(2):743-747.
13. Mornet E, Taillandier A, Domingues C, *et al*. Hypophosphatasia: a genetic-based nosology and new insights in genotype-phenotype correlation. *Eur J Hum Genet*. 2021;29(2):289-299.
14. Goseki-Sone M, Sogabe N, Fukushi-Irie M, *et al*. Functional analysis of the single nucleotide polymorphism (787T N C) in the tissue-nonspecific alkaline phosphatase gene associated with BMD. *J Bone Miner Res*. 2005;20(5):773-782.
15. Sogabe N, Oda K, Nakamura H, *et al*. Molecular effects of the tissue-nonspecific alkaline phosphatase gene poly-morphism (787T>C) associated with bone mineral density. *Biomed Res*. 2008;29(4):213-219.
16. Ermakov S, Toliat MR, Cohen Z, *et al*. Association of ALPL and ENPP1 gene polymorphisms with bone strength related skeletal traits in a Chuvashian population. *Bone*. 2010;46(5):1244-1250.
17. Masi L, Marini F, Franceschelli F, *et al*. Polymorphic variants of alkaline phosphatase gene correlate with clinical signs of adult hypophosphatasia? *Osteopor Int*. 2021;32(12):2461-2472.
18. Del Angel G, Reynders J, Negron C, Steinbrecher T, Mornet E. Large-scale in vitro functional testing and novel variant scoring via protein modeling provide insights into alkaline phosphatase activity in hypophosphatasia. *Hum Mutat*. 2020;41(7):1250-1262.
19. Bükmann Larsen P, Bjørn Skausig O, Jensen EA. Repeatedly low plasma alkaline phosphatase in a 56-year-old woman. A case of hypophosphatasia diagnosed in adulthood. *Pract Lab Med* 2018;11:19-22.
20. Tenorio J, Álvarez I, Riancho-Zarrabeitia L, *et al*. Molecular and clinical analysis of ALPL in a cohort of patients with suspicion of Hypophosphatasia. *Am J Med Genet*. 2017;173(3):601-610.
21. Henthorn PS, Raducha M, Fedde KN, Lafferty MA, Whyte MP. Different missense mutations at the tissue-nonspecific alkaline phosphatase gene locus in autosomal recessively inherited forms of mild and severe hypophosphatasia. *Proc Natl Acad Sci USA*. 1992;89(20):9924-9928.
22. Hofmann C, Girschick H, Mornet E, Schneider D, Jakob F, Mentrup B. Unexpected high intrafamilial phenotypic variability observed in hypophosphatasia. *Eur J Hum Genet*. 2014;22(10):1160-1164.

23. Reibel A, Manière M-C, Clauss F, *et al.* Orodental phenotype and genotype findings in all subtypes of hypophosphatasia. *Orphanet J Rare Dis*. 2009;4:6.
24. Mao X, Liu S, Lin Y, *et al.* Two novel mutations in the ALPL gene of unrelated Chinese children with Hypophosphatasia: case reports and literature review. *BMC Pediatr.* 2019;19(1):456.
25. Lefever E, Witters P, Gielen E, *et al.* Hypophosphatasia in adults: clinical spectrum and its association with genetics and metabolic substrates. *J Clin Densitom*. 2020;23(3): 340-348.
26. Gedmintas L, Solomon DH, Kim SC. Bisphosphonates and risk of subtrochanteric, femoral shaft, and atypical femur fracture: a systematic review and meta-analysis. *J Bone Miner Res*. 2013;28(8):1729-1737.
27. Nielson CM, Zmuda JM, Carlos AS, *et al.* Rare coding variants in ALPL are associated with low serum alkaline phosphatase and low bone mineral density. *J Bone Miner Res*. 2012;27(1):93-103.
28. Kharazmi M, Michaëlsson K, Schilcher J, *et al.* A genome-wide association study of bisphosphonate-associated atypical femoral fracture. *Calcif Tissue Int*. 2019;105(1):51-67.
29. Nguyen HH, van der Laarschot DM, Verkerk AJ, Milat F, Zil-likens MC, Ebeling PR. Genetic risk factors for atypical femoral fractures (AFFs): a systematic review. *JBMR Plus* 2018;2:11-11.