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A novel combination of biallelic *ALPL* mutations associated with adult hypophosphatasia: A phenotype-genotype association and computational analysis study

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ABSTRACT

Hypophosphatasia (HPP) is an inherited metabolic disorder that causes defective skeletal and dental mineralization. HPP exhibits a markedly heterogeneous range of clinical manifestations caused by dysfunction of the tissue-nonspecific isozyme of alkaline phosphatase (TNSALP), resulting from loss-of-function mutations in the *ALPL* gene. HPP has been associated with predominantly missense mutations in *ALPL*, and a number of compound heterozygous genotypes have been identified. Here, we describe a case of a subject with adult-onset HPP caused by a novel combination of missense mutations p.Gly473Ser and p.Ala487Val, resulting in chronic musculoskeletal pain, myopathy, persistent fatigue, vomiting, and an uncommon dental phenotype of short-rooted permanent teeth. Pedigree and biochemical analysis indicated that severity of symptoms was correlated with levels of residual ALP activity, and co-segregated with the p.Gly473Ser missense mutation. Bioinformatic analysis to predict the structural and functional impact of each of the point mutations in the TNSALP molecule, and its potential contribution to the clinical symptoms, revealed that the affected Gly473 residue is localized in the homodimer interface and predicted to have a dominant negative effect. The affected Ala487 residue was predicted to bind to Tyr479, which is closely located the N-terminal α-helix of TNSALP monomer 2, suggesting that both changes may impair dimer stability and catalytic functions. In conclusion, these findings assist in defining genotype-phenotype associations for HPP, and further define specific sites within the TNSALP molecule potentially related to neuromuscular manifestations in adult HPP, allowing for a better understanding of HPP pathophysiology.

1. Introduction

Hypophosphatasia (HPP) is a rare hereditary disorder caused by loss-of-function mutations in the *ALPL* gene (MIM*171760) that encodes the tissue-nonspecific isozyme of alkaline phosphatase (TNSALP, UniprotKB# P05186). This inborn error-of-metabolism is characterized by defective skeletal and dental mineralization, and other less well understood complications resulting from deficient TNSALP activity and consequent accumulation of TNSALP substrates [1,2]. Clinical manifestations of HPP are markedly heterogeneous, ranging in severity from stillbirth with no skeletal mineralization to spontaneous fractures in adult life, and sometimes limited to premature exfoliation of primary teeth in the absence of other symptoms [1–4]. Clinical forms of HPP are classified according to the age of onset and severity of symptoms and include: lethal perinatal and prenatal benign HPP (MIM#241500); infantile (MIM#241500) and pseudo-HPP (resembles infantile HPP except serum ALP activity is within the normal range); severe and mild childhood/juvenile HPP (MIM#241510); adult HPP; and odonto-HPP (MIM#146300) [1–4].

Manifestations of HPP may include defective bone mineralization

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with bone deformities, osteomalacia and osteopenia, fractures, short stature/rickets, premature craniosynostosis, arthropathy, premature loss of deciduous teeth, enamel and dentin defects, and seizures [1–3,5]. The clinical suspicion of HPP is confirmed by low levels of serum alkaline phosphatase activity (ALP) and genetic screening of the *ALPL* gene [1,6,7]. The developmental bone defects described above are typical of more severe and earlier onset forms of HPP. Clinical presentation of adult-onset HPP most often features bone fractures in the legs or feet, musculoskeletal pain, myopathy (including hypotonia, muscle weakness, and muscle pain), and other symptoms similar to fibromyalgia, a condition that features muscle pain, sleeping problems, persistent fatigue, and even chronic depression [4,8–13]. Diagnosis of HPP in adulthood may be challenging due to absence of distinctive characteristics of HPP, as well as a predominance of signs and symptoms frequently found in other conditions common in the general population with a tendency to become more prevalent with age [4,8–13]. Dental manifestations are nearly universal in all forms of HPP, and premature tooth loss is commonly the first and sometimes the only visible symptom of mild forms of HPP, and therefore may assist in diagnosis [14]. Premature tooth loss in childhood has sometimes only been recognized retrospectively after other signs of HPP manifested in adulthood. In a retrospective study, Mori et al. found that diagnosis of mild forms of HPP was delayed on average 46 years after premature loss of deciduous teeth, and 27 years after the first fracture or onset of a major problem in the permanent dentition [11]. Furthermore, it is becoming better established that patients classified with odonto-HPP can later manifest a considerable burden of disease in adult life, including fractures and musculoskeletal pain [11].

At present, strong genotype-phenotype associations have not been established for HPP. Individuals diagnosed with adult HPP have been reported to exhibit low serum ALP levels and harbor single or compound heterozygous *ALPL* mutation(s), though may display mild manifestations or be asymptomatic at the time of study [7–11,13,15–17]. Severity and broad-ranging expressivity of HPP manifestations involve a number of genetic and molecular factors, including strong allelic heterogeneity of *ALPL* and different compound heterozygous genotypes, distinct inheritance patterns [6,7], dominant negative effects [17–20], incomplete gene penetrance of dominant forms, parental bias of transmission observed in the prenatal benign form of HPP [6,21], and presumed interactions with modifier genes [22]. At least 388 distinct *ALPL* mutations have been described in association with HPP (Mornet E, January 22, 2019; http://www.sesep.uvsq.fr/03_hypo_mutations.php), and among them, about 73% represent missense mutations. Several of the missense mutations have been demonstrated to affect important residues for protein stability and function [18–20,22–24]. In this context, gene segregation and bioinformatic analysis may continue to provide insights into HPP genotype-phenotype associations. Here, we present a case of an individual carrying a novel combination of *ALPL* compound heterozygous missense mutations and diagnosed with adult HPP featuring primarily neuromuscular manifestations.

2. Methods

2.1. Clinical evaluation

The study protocol was approved by the Piracicaba Dental School - University of Campinas Institutional Review Board (IRB# 88910618.0.0000.5418), and the study was conducted in compliance with the recommendation of the World Medical Association Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects. A written informed consent or assent was obtained for all individuals enrolled to report their clinical data, retrospective medical histories, and genetic information. The proband was a 20-year-old Caucasian female with suspected adult-onset HPP based on low serum ALP levels, who exhibited additional nonspecific clinical symptoms. She was referred for genetic testing to confirm the diagnosis of HPP. At the age of 22, due to increased tooth mobility, the proband was referred by her general dentist to the Piracicaba Dental School (University of Campinas, Brazil). The proband's medical and dental histories were assessed, while other family members, including her mother, maternal grandfather, and three half-siblings (one by the mother and two by the father), were also evaluated and genetically analyzed in order to identify inheritance and genotype-phenotype association patterns. Genotypic and phenotypic evaluations could not be performed for her biological father, who was deceased. Oral examinations and dental radiographs (panoramic and periapical) were obtained for all subjects evaluated.

2.2. Genetic analysis

Genetic mutation analysis was performed by sequencing the coding regions of the *ALPL* gene, including intron-exon borders [25]. The twelve exons of *ALPL* were amplified by polymerase chain reaction (PCR), followed by bi-directional sequencing using a capillary electrophoresis Sanger sequencing instrument (ABI3500 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA). *ALPL* genetic variants identified in the proband were confirmed by Sanger sequencing and screened in other family members in order to verify the segregation pattern of *ALPL* mutations. Genetic analysis methods followed a previously reported protocol [25]. Briefly, genomic DNA was isolated from the buccal mucosa cells and used as a template to amplify regions of the *ALPL* gene by PCR, using primers: forward 5′-CCCACAGCTCACAACA ACTA-3′ and reverse 5′-TGTGGGAAGTTGGCATCTG-3′. Sanger sequencing of PCR products was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit and migrated on capillary 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequence similarity was assessed using BLASTN [26] and the presence or absence of the heterozygous mutations, NM_000478.5:c.1417G > A (p.Gly473Ser) and NM_000478.5:c.1460C > T (p.Ala487Val), was verified by electropherogram.

2.3. Residue conservation analysis

Multiple species amino acid sequence alignment for TNSALP was performed using Clustal Omega (European Bioinformatics Institute, EMBL-EBI; http://www.ebi.ac.uk/Tools/msa/clustalo/). The sequence logo for TNSALP was determined using WebLogo analysis [27]. Orthologous TNSALP protein sequences from 19 vertebrate species were used for the multiple sequence alignment and sequence logo construction, including: human (*ALPL*: NP_000469.3; *ALPP*: NP_001622.2; *ALPI*: _001623.3; *ALPPL*: NP_112603.2); monkey (XP_014985698.1); chimpanzee (XP_016811317.1); gorilla (XP_004024892.1); orangutan (XP_009232332.1); rat (XP006239198.1); mouse (NP_031457.2); domestic guinea pig (XP_013009513.1); dolphin (XP_007459278.1); cat (NP_001036028.1); dog (XP_005617271.1); cow (NP_789828.2); horse (XP_014592921.1); goat (XP_017910721.1); sheep (XP_012008214.1); rabbit (XP_017201978.1); chicken (NP_990691.1); zebrafish (NP957301.2); and tilapia (XP_003447731.1) sequences.

2.4. Homology modeling for TNSALP

Three-dimensional (3D) models for native and mutant TNSALP proteins were built based on the previously determined crystal structure of human placental alkaline phosphatase (PDB ID: 1EW2) [28] using SWISS-MODEL software [29]. These models were aligned, visualized, and analyzed using the open source PyMOL software (PyMOL Molecular Graphics System, Version 1.7.4, Schrödinger, LLC). Web-based interactive tool PDBePISA was used to allow a detailed analysis of surface (solvent accessible and buried residues), interfaces and assemblies for PLAP crystal structure (PDB: 1EW2). In order to visualize details of the intramolecular and intermolecular interactions, 3D stick

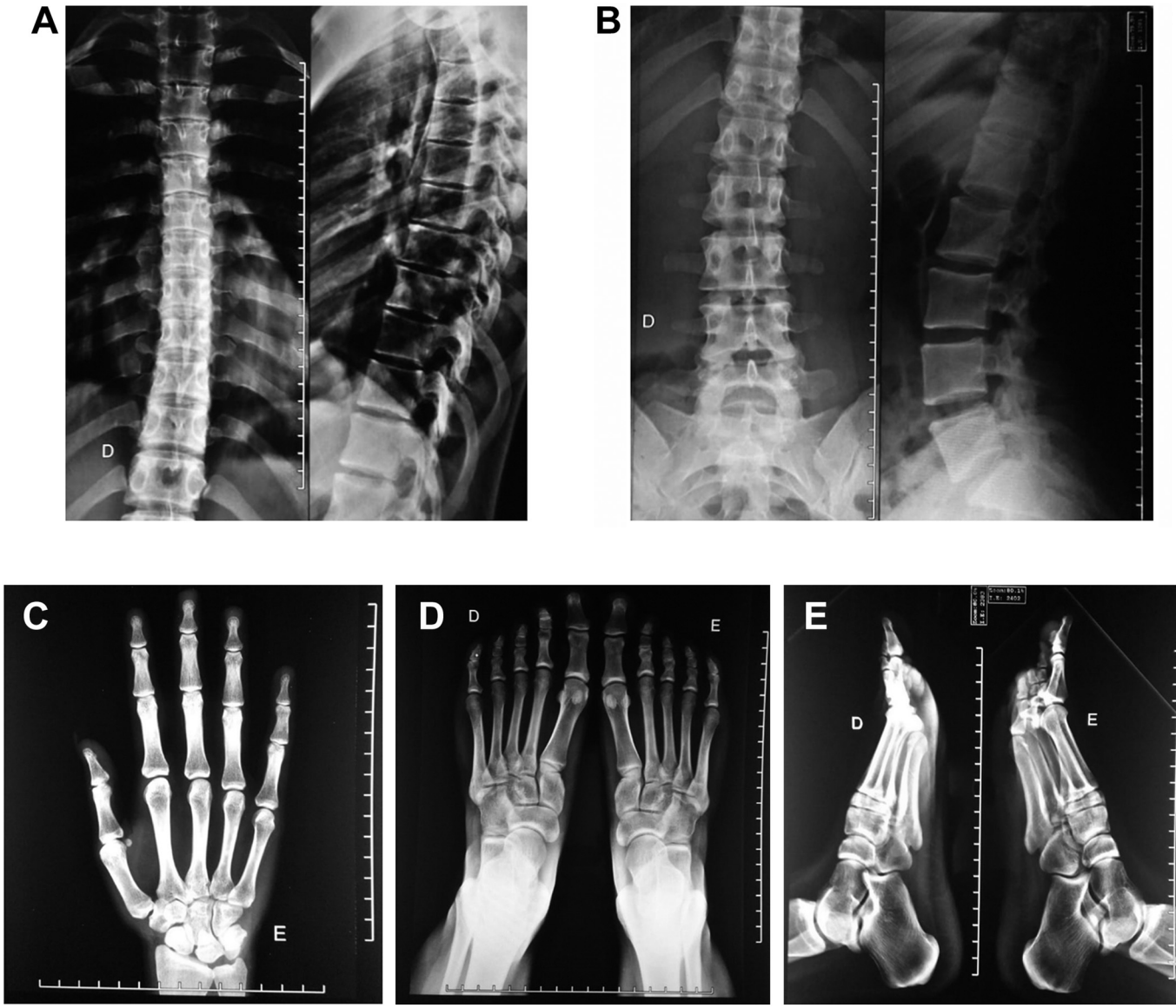

Fig. 1. Minimal skeletal effects of HPP on the proband. Images were acquired before asfotase alfa treatment, when the proband (III.4) was 20 years old. Lateral and dorsal radiographs of the spine from (A) cervical/thoracic and (B) lumbar/sacral regions reveal discrete lumbar scoliosis (indicated by arrow). Vertebral bodies were preserved and no significant reductions of intervertebral disc spaces were observed. (C) A radiograph of the left hand shows no evidence of metaphyseal fraying or characteristic "tongues" of radiolucency. Radiographs showing frontal and lateral views (D and E, respectively) of the feet show no signs of fractures or pseudo-fractures, with an absence of gross bone irregularities and pathological calcifications, and articular spaces that appear normal and free of ectopic calcification. The skeletal age of the proband (III.4) was estimated to be 17 years according to the Greulich–Pyle (GP) method.

models from native or mutant TNSALP dimer, showing the hydrogen bond and inter-atom distances, were constructed using the modeling tool, Swiss-Pdb Viewer [30].

3. Results

3.1. Medical and dental history of the proband

The proband was a 20-year-old Caucasian female who reported symptoms similar to fibromyalgia over the course of several years, including chronic musculoskeletal pain (bone, muscle, and joint pain), myopathy (muscle weakness), muscle tenderness, spasms, persistent fatigue, malaise, weakness, excessive tiredness, sleeping problems, apathy, anxiety, mood swings, dizziness, fainting, and nausea and vomiting. No bone irregularities, pathological calcifications, or evidence of fractures or pseudo-fractures were found in proband's hand and foot radiographs, while the proband's spine radiographs showed only a discrete dorsal lumbar scoliosis (Fig. 1). Bone mineral density, assessed in the proband's spine (L1-L4 region; 1.162 g/cm^2, *Z*-score = 0.1) and femur (0.845 g/cm^2, Z-score = −1.3), were within the normal limits expected for a young population with the same age, sex, ethnicity, weight, and height. Bone age at 20 years old, as assessed by the Greulich-Pyle method (a common radiographic method to determine skeletal age based on appearance of the hand compared to established standards), was compatible with 17 years, indicating a slight delay in skeletal development and growth. The proband (III.4) and her family pedigree (described below) are summarized in Fig. 2A.

Biochemical blood testing revealed that the proband exhibited very low serum ALP levels of 6 U/L (normal range for adult: 40–150 U/L), and low 25-hydroxyvitamin D at 27.1 ng/mL (normal range for adult: 30–100 ng/mL). Pyridoxal phosphate (PLP) serum levels were not measured. Normal serum levels were found for calcium (9.5 mg/dL; normal range for adult: 8.60–10.20 mg/dL), phosphorus (3.9 mg/dL; normal range: 2.7–4.5 mg/dL), creatinine (0.75 mg/dL; normal range:

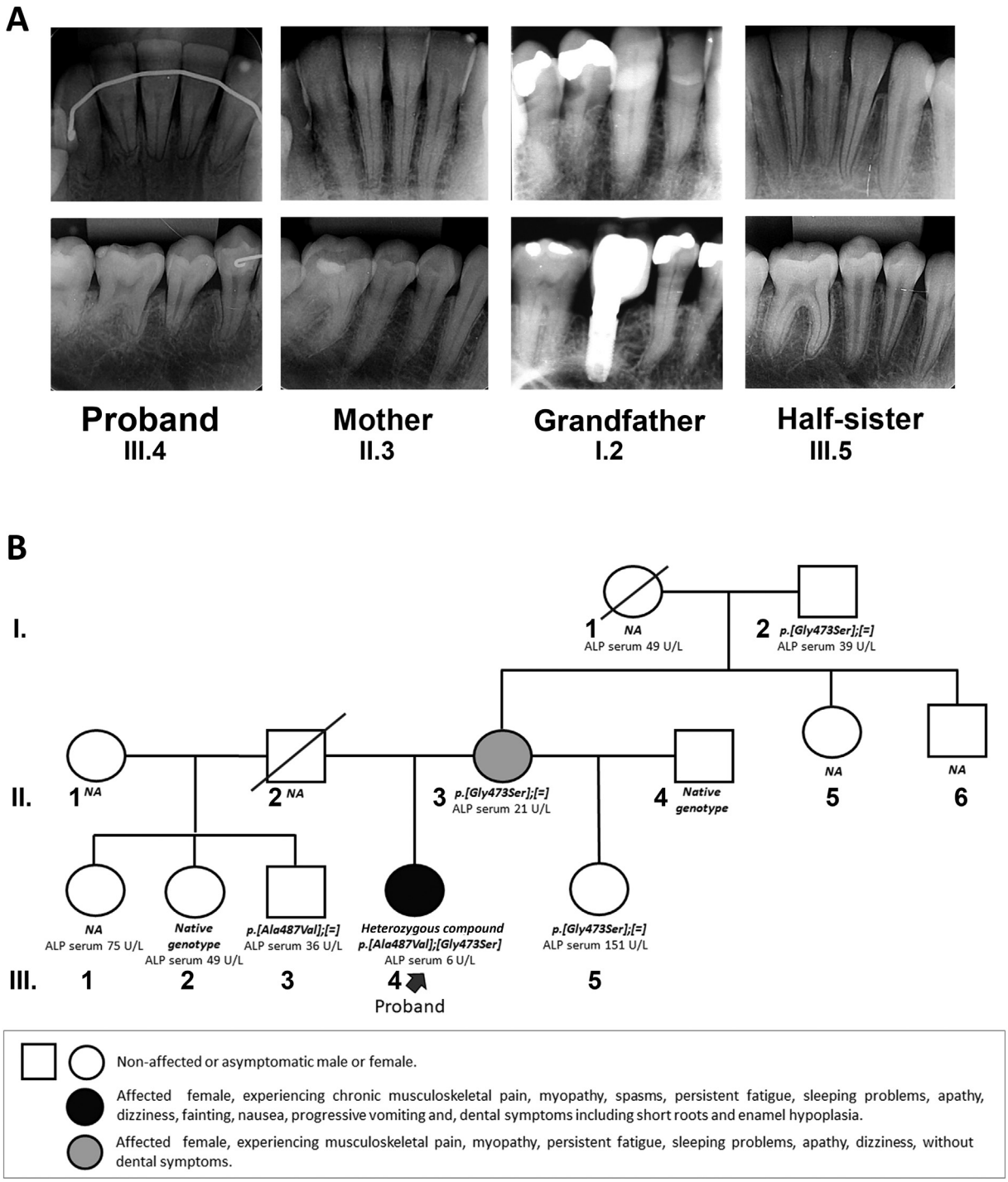

Fig. 2. Medical and dental findings in the pedigree. (A) Representative intraoral radiographs showing short-rooted permanent teeth and alveolar bone loss in the proband (III.4), and normal dentoalveolar tissues in her mother (II.3), grandfather (I.2), and half-sister (III.5). (B) Pedigree chart covering three generations. Segregation patterns of genotypes (p.Gly473Ser and/or p.Ala487Val) and phenotype (serum ALP levels and clinical and dental findings) for proband III.4 (arrow) and her maternal and paternal relatives are summarized. N/A: not clinically or genetically evaluated.

0.50–0.90 mg/dL), creatine phosphokinase (113 U/L; normal range: 26–192 U/L), and parathyroid hormone (36 pg/mL; normal range: 15–65 pg/mL).

Dental assessment revealed signs of enamel hypoplasia and an abnormal dark yellow enamel hue, excessive tooth mobility, and a slight atrophy of alveolar bone. Intraoral examination revealed no signs of gingivitis, gingival recession, bleeding on probing, or swelling. Periodontal probing showed normal attachment levels and no sign of periodontal pocketing. Radiographic examination indicated unusually short roots on several permanent teeth (Fig. 2A). Upon collection of medical history information, the proband's mother recalled that primary anterior teeth exfoliated slightly early (approximately at age 5 years) and were fully rooted.

3.2. Family history and pedigree evaluation

Medical histories were assessed for available members of the proband's immediate and extended family (summarized in the pedigree

diagram in Fig. 2B). Similar to the proband, the mother (II.3; 45 years old) complained of chronic musculoskeletal pain (bones, muscles, back, and joints), myopathy (muscle weakness), persistent fatigue (malaise, weakness, feeling tired), paresthesia (tingling or feeling cold in the feet or hands), nausea and constipation, dizziness/vertigo, sleeping problems (difficulty falling asleep and drowsiness), apathy, anxiety, mood swings, and irritability and headache (Fig. 2, II.3). She reported no history of fractures or tooth abnormalities. Previous assessments, including electroneuromyography testing and ultrasound of wrist elbow, forearm, and upper limbs were unremarkable (data not shown). At age 42 years old, she was diagnosed with fibromyalgia by a rheumatologist. Biochemical analysis revealed that proband's mother (II.3) exhibited low serum ALP levels of 21 U/L (normal range for adult: 40–150 U/L). Clinical and radiographic assessments indicated no alveolar bone or dental pathologies, and tooth roots appeared normal (Fig. 2A). An evaluation could not be performed for the proband's biological father, who was deceased.

Evaluations of the proband's maternal grandfather and three half-siblings (one by the mother and two by the father) were also performed. Biochemical analysis revealed that proband's grandfather (I.2) had borderline low ALP serum levels of 39 U/L (normal range for adult: 40–150 U/L), whereas ALP serum levels were within normal limits (151 U/L; normal range for age: 141–460 U/L) for the proband's half-sister (III.5) (Fig. 2). Neither exhibited dentoalveolar abnormalities (Fig. 2A). On the paternal side, two out of the three half-siblings were evaluated. Both half-siblings presented no remarkable medical or dental histories. The proband's 29-year-old half-brother (III.3) presented slightly lower than normal serum ALP levels of 36 U/L (normal range for adult: 40–150 U/L), whereas the proband's half-sister (III.2) presented serum ALP levels in the normal range (49 U/L and 75 U/L) (Fig. 2B).

3.3. Genetic analysis and diagnosis

Genetic analysis was performed for the proband and several members of her family. The proband (III.4) exhibited heterozygous missense mutations in *ALPL*: c.1417G > A (p.Gly473Ser) and c.1460C > T (p.Ala487Val), both located in exon 12 (Fig. 3A). Based on her medical and dental history, pedigree evaluation, low serum ALP levels, and *ALPL* mutations, she was diagnosed with adult HPP. The proband's mother (II.3), who was heterozygous for only the p.Gly473Ser *ALPL* mutation, was identified as a symptomatic carrier, exhibiting low ALP levels and relatively milder symptoms. The proband's maternal grandfather (I.2; 83 years old) and half-sister by her mother (III.5; 12 years old), were defined as asymptomatic carriers of the p.Gly473Ser heterozygous *ALPL* mutation. To date, they have not experienced any of the symptoms reported by the proband and her mother, and presented no history of fractures or other clinical manifestations of HPP. On the paternal side, the proband's half-brother (III.3) carried the p.Ala487Val heterozygous mutation and was identified as an asymptomatic carrier, while the half-sister (III.2) with normal ALP did not carry the mutation. This finding verified that the p.Ala487Val mutation was inherited from the father's side.

3.4. Bioinformatic analysis of ALPL mutations

In order to better understand the potential mechanisms underlying TNSALP loss-of-function and the broad expressivity of HPP manifestations in this cohort, we used bioinformatic approaches to assess p.Gly473Ser and p.Ala487Val mutations by amino acid residue conservation analysis, 3D homology modeling, and dimer-interface analysis. Multiple sequence alignment between *ALPL* and paralogous phosphatases [including placental alkaline phosphatase (*ALPP*), intestinal alkaline phosphatase (*ALPI*), and placental-like alkaline phosphatase (*ALPPL*)] or between *ALPL* and orthologous sequences from 19 different vertebrate species additionally revealed perfect conservation of both Gly473 and Ala487 residues (Supplementary Fig. 1). Next, we built a logo sequence representing the conservation of TNSALP amino acid residues across species, and *ALPL* missense mutations (homozygous, heterozygous or heterozygous compound) previously reported for adult HPP patients (Supplementary Table 1), including p.Gly473Ser and p.Ala487Val (Fig. 3B). Mutations were mapped to the 2D sequence with annotations indicating the major functional regions of the TNSALP protein. It was found that the majority of single heterozygous mutations associated with adult HPP were located within the homodimer interface (also the case for p.Gly473Ser and p.Ala487Val), active site vicinity, and metal-binding sites, while the calcium binding domain region was relatively less frequently affected (Fig. 3B). Furthermore, 3D modeling analysis showed that Ala487 residue is located in the N-terminal α-helix adjacent to the interface region, whereas the Gly473 residue is located in the coil structure within the center of the homodimer interface region, suggesting an impact on the enzyme catalytic function and a potential disruption of dimerization (Fig. 4).

Intramolecular and intermolecular contact analyses were performed to better predict the molecular effects of the *ALPL* mutations. Gly473 is bound covalently with Val474 to establish hydrogen bonds with the Leu97 residue (Fig. 5A, B). Substitution of Gly473 by a Ser473 residue is predicted to result in a new hydrogen bond between Ser473 and Val474 (Fig. 5B). Furthermore, the Val474 residue from monomer 1 was predicted to establish two hydrogen bonds with the Arg86 residue from monomer 2 (Fig. 5A). These findings support that the Gly473 to Ser473 substitution promotes conformational changes that may affect dimer formation or stability. A slight change in the hydrogen bond distances was observed as result of the Ala487 to Val487 substitution (Fig. 5A, C). However, Ala487 was predicted to form a hydrogen bond with Val483 and with the Ala51 residue in the coil structure that precedes the N-terminal α-helix (Fig. 5A). Additionally, Ala487 in monomer 1 establishes an indirect ligation with residues that are in close proximity to the N-terminal α-helix of monomer 2 (Fig. 5A, C), supporting that the Ala487 to Val487 substitution may affect the dimer interface quality.

4. Discussion

We describe here a novel combination of biallelic p.Gly473Ser and p.Ala487Val *ALPL* missense mutations associated with adult HPP in a young woman. The proband exhibited low ALP levels, dental abnormalities, and absence of pathologic fractures, however, suffered from a wide range of gastrointestinal and neuromuscular manifestations, including musculoskeletal pain, persistent fatigue, dizziness, fainting, and nausea and progressive vomiting. The genotype-phenotype association in the proband's pedigree suggested that both mutations contributed individually to low serum ALP levels, but that musculoskeletal symptoms co-segregated with the p.Gly473Ser mutation found in the proband and her mother. The proband experienced more severe musculoskeletal symptoms than her mother, exhibited the most severe reduction in ALP levels, and was the only pedigree member exhibiting gross dental abnormalities, suggesting that the combination of heterozygous *ALPL* mutations increased the severity of HPP in this individual. Both affected residues were highly conserved and mapped to exon 12 of the *ALPL* gene. The mutations localized to the dimer interface region, and bioinformatic analysis suggested molecular disruptions within and between TNSALP monomers resulting from altered amino acid properties. In summary, this study introduces a new heterozygous combination of *ALPL* mutations and links them to an uncommon dental phenotype, and relatively severe neuromuscular and gastrointestinal manifestations that negatively affected quality of life.

4.1. HPP genotype-phenotype associations

Severe forms of HPP are rare, with an estimated prevalence of 1/300,000 in the European population, 1/450,000 in Japan, 1/100,000 in

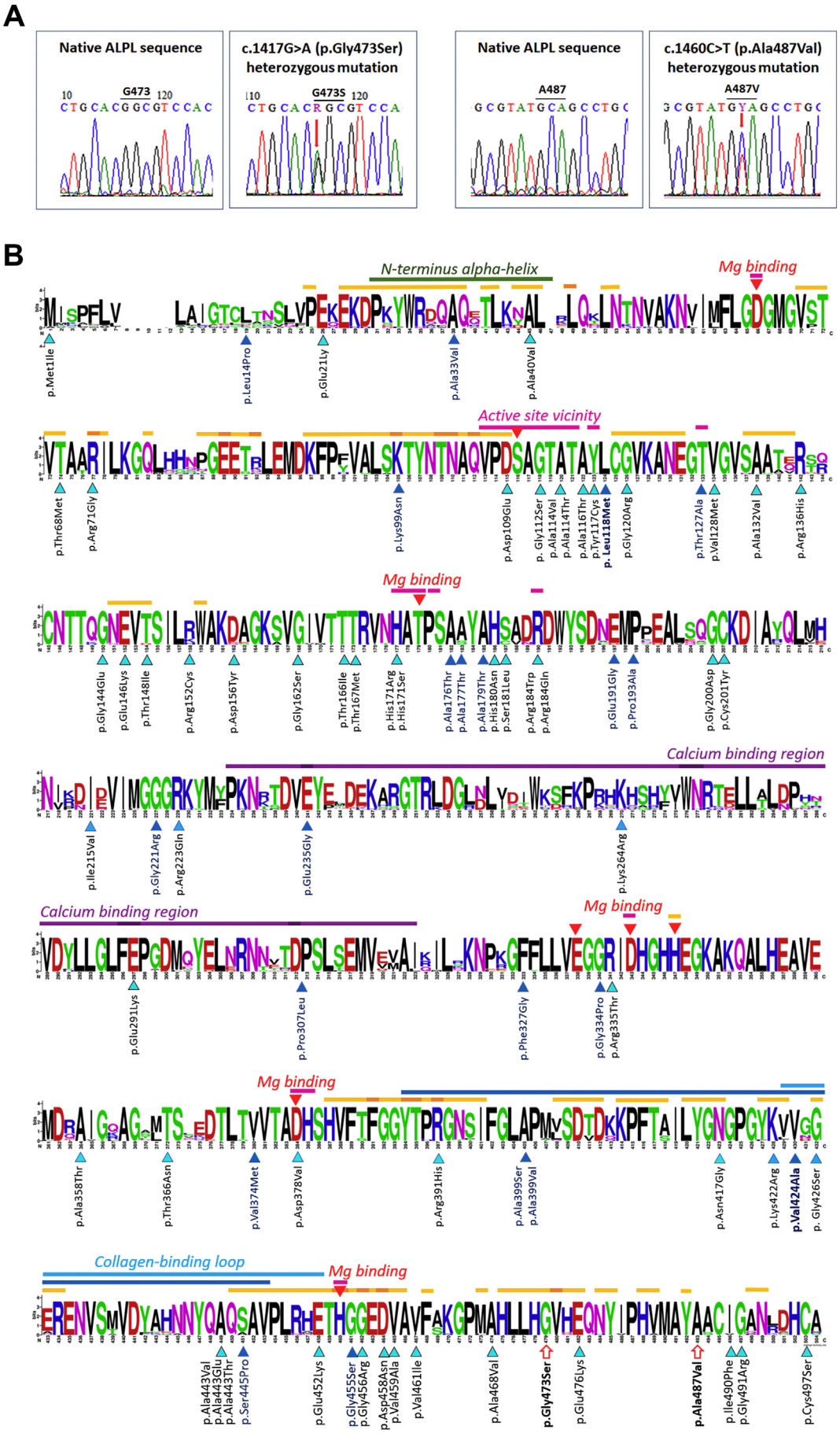

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Fig. 3. Genetic analysis of *ALPL* mutations. (A) Electropherograms indicating native and heterozygous sequences for *ALPL* for the substitution of G for A at position 1417-nt (c.1417G > A), which results in a change of Glycine 473 to Serine (p.Gly473Ser), or substitution of C for T at position 1460-nt (c.1460C > T), resulting in a change of Alanine 487 to Valine (p.Ala487Val). (B) Representation of evolutionary conservation of TNSALP residues 1 to 498. The data to create the TNSALP logo sequence include orthologous TNSALP protein sequences from 19 different vertebrate species. The horizontal axis indicates the position of the amino acids in the TNSALP protein sequence and height of each individual symbol reflects prevalence of mutations at the given position. TNSALP functional regions, including active site vicinity/valley (pink bar: Asp60, Asn102, Pro108-Thr115, Tyr117, Thr127, His171-Thr173, Ala176, Arg184, Asp337, Asp378, His 379, His454) and active site [red inverted triangle: phosphoserine intermediate (Ser110)], metal-binding sites (red inverted triangles: Zn1: His341, His454, Asp337, Ser110-PO_3; Zn2: Asp60, His379, Asp378, Ser110-PO_3 and Mg: Asp60, E332, Thr173, Ala172), calcium binding domain (violet bar: Lys229 – Gln318; into this domain the Glu235, Trp270, Asn271, Phe290, Glu291, Asp306), residues directly involved in calcium-binding, (dark violet bar: Glu235, Phe290, Glu291, Asp306, Trp270, Asn271), crown domain (dark blue bar; Tyr388-Pro448 residues), collagen binding (light blue bar: 422–452 residues), N-terminus α-helices (green bar: Pro26 – Glu42 residues), close dimer interface residues (orange bar), and regions involved in monomer-monomer interaction (dark orange bar: Leu43, Arg71, Glu83, Arg86, Lys99, Thr103, Asn104, Gln106, Phe385, Tyr388, Thr389, Arg391, His454, Gly456, Asp458, Val474), are indicated in the figure. The locations of the p.Gly473Ser and p.Ala487Val mutations are indicated by red outlined arrows and bold text. The locations of the heterozygous *ALPL* mutations associated with adult-HPP phenotype are indicated by light blue triangles and text in black, while compound heterozygous mutations are indicated by blue triangles and text in blue. Homozygous *ALPL* mutations resulting in adult HPP are indicated by blue triangles and text in bold blue. The genotypes and references related to each mutation represented in the Weblogo sequence are detailed in the supplementary table. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the Toronto region of Canada, and 1/2500 in Mennonite families in Manitoba, Canada [31]. On the other hand, prevalence is difficult to accurately estimate for milder forms of HPP, including childhood, adult, and odonto-HPP, because of incomplete penetrance [6], a wide spectrum of severity, heterogeneity, and variable expressivity of clinical manifestations, as well as a large number of cases that likely remain undiagnosed and/or misdiagnosed [7–9,11,16]. Clinical manifestations of milder forms of HPP feature more nonspecific signs and symptoms, including fractures, osteomalacia, and musculoskeletal pain, that can be easily misdiagnosed in the absence of biochemical and genetic information [7–9,11,16]. While genotype-phenotype associations have been established for some HPP-causing *ALPL* mutations that were directly investigated, the majority are not well understood in terms of location and type of mutations and biochemical, musculoskeletal, and neurological effects.

Genetic analysis can be helpful for diagnosis and genetic counseling in families that carry HPP-causing *ALPL* mutations. Two mutations were identified in the proband, both located in *ALPL* exon 12: p.Gly473Ser and p.Ala487Val. Mutation of Gly473 has been described three times before, twice associated with another *ALPL* mutation or deletion in individuals with severe perinatal HPP [32,33], and once as the sole heterozygous mutation in an adult with mild HPP [7]. Mutation of Ala487 has been described once before, in combination with an *ALPL* deletion associated with perinatal HPP [22]. In spite of very low ALP levels, the proband exhibited a milder manifestation of HPP marked by dental defects and adult onset musculoskeletal symptoms. The musculoskeletal manifestations were shared with her mother who carried only the p.Gly473Ser mutation and had less severely reduced ALP levels. The proband's maternal grandfather and half-sister by her mother also carried the p.Gly473Ser mutations, but appeared to be asymptomatic carriers as ALP levels were low-borderline normal and dental, gastrointestinal, and musculoskeletal manifestations were not present. Members of the father's family harbored the p.Ala487Val mutation, which was associated with mild reduction of ALP but no overt signs of HPP. This is consistent with a previous report where an *in silico* approach predicted p.Ala487Val to be deleterious but without a dominant negative effect [22].

Consistent with our observations, a previous study reported that adults with persistently low ALP levels are likely to carry a potential pathogenic variant in the *ALPL* gene, and may harbor previously unrecognized HPP [34]. Similar to the proband described here, McKiernan and colleagues (2017) found that while these individuals initially lack some or all recognizable clinical manifestations of HPP, biochemical and radiographic findings in these individuals may eventually fall within the spectrum recognized as the adult form of HPP [34]. In addition, this study reported that not all subjects carrying potentially pathogenic *ALPL* gene variants showed elevation in TNSALP substrates plasma pyridoxal-5-phosphate (PLP) or in urine phosphoethanolamine (PEA) [34]. Genotype and phenotype segregation and biochemical data in the family described here indicated that the proband's severity of gastrointestinal and neuromuscular symptoms was associated with low levels of residual ALP activity.

A number of studies have reported the impact of *ALPL* missense mutations on enzyme activity, dimer stability, allosteric proprieties, uncompetitive inhibition, substrate specificity, and catalytic activity, cell localization, and degradation of the mutant proteins, contributing to greater understanding of genotype-phenotype relationships [18–20,23–25]. In the current investigation, we employed several bioinformatic approaches, including 3D modeling and inter- and intramolecular interaction analyses to predict the impact of the *ALPL* mutations on TNSALP structure and function, and potentially make sense of the biochemical and clinical manifestations. The affected Gly473 residue was localized to the homodimer interface and predicted to have a negative dominant effect on protein function. According to previous studies, residues located in the activity site vicinity, crown domain, and homodimer interface are expected to exert a dominant negative effect by impairing dimerization and/or allosteric properties of the enzyme [18,22]. This could explain, at least in part, the variable expressivity observed among carriers of the single heterozygous mutation. In addition, the severity of phenotype may vary depending on its combination with other genetic alterations. Ala487 also localized to the homodimer interface, and although p.Ala487Val was not predicted to have a dominant negative effect, we propose that in combination with p.Gly473Ser may affect the quality of the dimer interface and thus impair TNSALP allosteric proprieties and catalytic function.

The combined evidence supports that the p.Gly473Ser mutation has a dominant negative effect and is associated with musculoskeletal effects, but that the combination of heterozygous missense mutations likely increased severity of effects in the proband, causing a more dramatic reduction in ALP levels and also contributing to dental abnormalities, neurologic (fainting) and gastrointestinal manifestations (vomiting and abdominal pain) recognized in the proband. Previous reports confirmed that moderate and mild HPP forms may result from single heterozygous mutations with a dominant negative effect, or from heterozygous compound genotypes [17,18]. These observations provide new insights regarding genotype-phenotype association as this combination of missense mutations has not been reported to date (Mornet E, April 8 22,2019; http://www.sesep.uvsq.fr/03_hypo_mutations.php).

4.2. Asfotase alfa treatment

During the course of this study, the proband was treated with TNSALP enzyme replacement therapy, asfotase alfa (commercial name Strensiq™; Alexion Pharmaceuticals) by her physician. Asfotase alfa is a hydroxyapatite-targeted recombinant form of TNSALP approved by U.

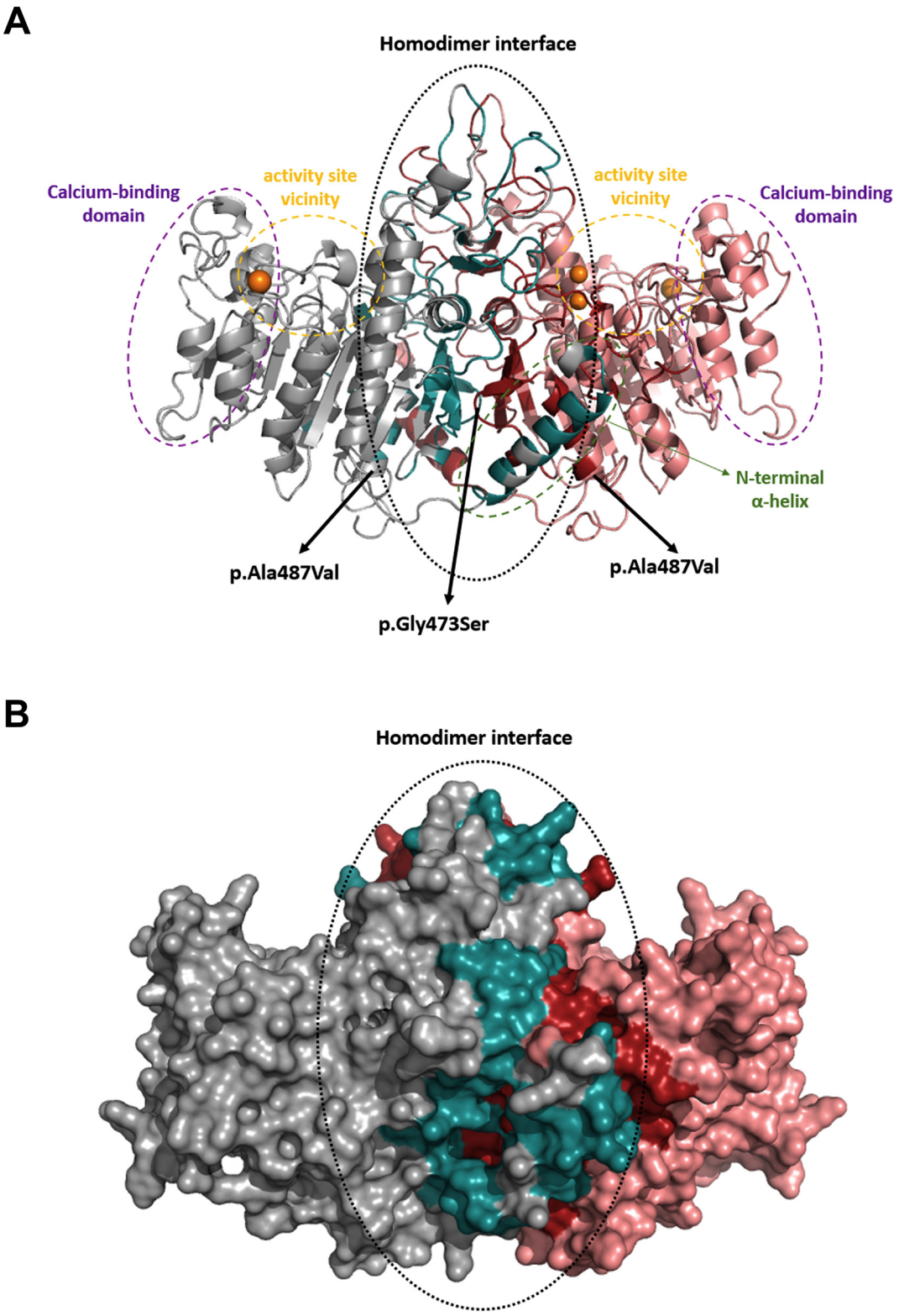

Fig. 4. 3D location of TNSALP substitutions. (A) Cartoon representation and (B) surface model showing monomer 1 (light gray) and monomer 2 (salmon) of the TNSALP homodimer, with key functional domains labeled. Residues close to the monomer-monomer interface are highlighted in cyan in monomer 1 and in red in monomer 2. Localization of p.Gly473Ser and p.Ala487Val substitutions are indicated by arrows. The images were generated using the PyMol software. Gly473, a non-polar aliphatic neutral residue, which can fit into hydrophilic or hydrophobic environments, was located in the coil structure at the dimer interface. Gly473 is predicted to be a solvent-accessible residue (molecule surface), whereas Ser473 is a polar neutral residue containing a hydroxymethyl group. Ala487 is non-polar aliphatic neutral residue (side chain methyl group), located in the α-helix and predicted to be an inaccessible residue (buried within the molecule structure). The substituted Val487 residue (side chain isopropyl group) presents chemical proprieties similar to Ala487. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

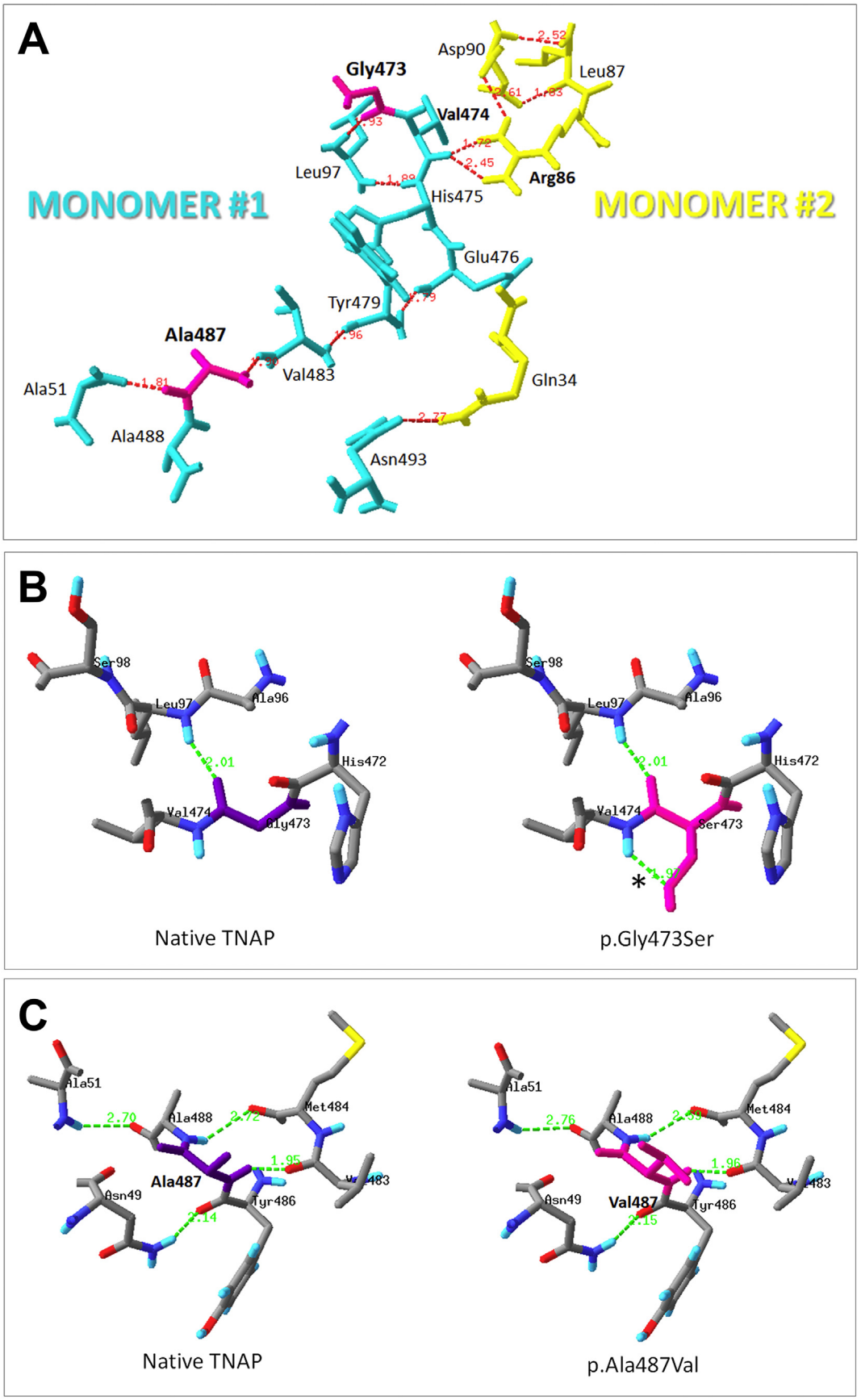

Fig. 5. Intramolecular alterations arising from *ALPL* mutations. (A) Intermolecular interactions are shown between monomer I (cyan) and monomer II (yellow). Hydrogen bonds (dashed red lines) and their distances are indicated. (B) Intramolecular interactions established by native Gly473 and mutant Ser473 residues are indicated. A predicted new interaction between Ser473 and Val474 is indicated by an asterisk. (C) Intramolecular interactions established by native Ala487 and mutant Val487 residue are indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

S. Food and Drug Administration and indicated for use in life-threatening perinatal/infantile and severe childhood or juvenile-onset cases of HPP in the United States, Canada, Europe, and Japan [35,36]. The efficacy and safety of asfotase alfa were evaluated in preclinical mouse studies and 5 prospective, open-label, phase 2, multinational clinical studies in infants and adolescents with perinatal, infantile, or childhood HPP [35]. Treated children exhibited dramatic improvements in survival, bone mineralization, growth, respiratory function, and mobility [35]. More recently, a multinational, randomized, open-label study in adults and adolescents with pediatric-onset HPP, demonstrated that treatment with asfotase alfa may result in improved functional abilities, including improvement of gross motor function and muscle strength, and suggested that these improvement may be associated with the normalization of circulating TNSALP substrates levels observed in these patients [37]. For the proband, treatment with asfotase alfa for 4 months resulted in self-reported overall improvements including reduced musculoskeletal discomfort, gastrointestinal problems (nausea, vomiting and abdominal pain), and neurological manifestations (fatigue, dizziness and fainting). However, therapy was discontinued after a severe anaphylactic reaction. By 6 months after enzyme replacement was discontinued, ALP serum levels again plummeted to below normal range (12 U/L), the proband's clinical improvements had disappeared, and neuromuscular and gastrointestinal manifestations had returned. Although rare, hypersensitivity reactions, including signs and symptoms consistent with anaphylaxis, have been described in association with asfotase alfa [35]. Other signs of hypersensitivity reactions reported included difficulty breathing, nausea, periorbital edema, dizziness, vomiting, fever, headache, irritability, chills, skin erythema, rash, pruritus, and oral hypoesthesia [35]. At present, potential mechanisms for neuromuscular and gastrointestinal manifestations of HPP remain unknown, and further studies are necessary in order to determine the effectiveness of enzyme replacement therapy to improve these aspects of HPP.

4.3. Manifestations of adult HPP in this cohort

The proband and her mother described in this report experienced adult-onset HPP with an array of clinical manifestations, including chronic musculoskeletal pain, fatigue, myopathy, vomiting, and fainting, but with no history of clinically evident skeletal involvement or fractures. A number of physicians were consulted unsuccessfully until low ALP levels were recognized and genetic testing revealed heterozygous *ALPL* missense mutations. The proband's mother suffered from a similar array of musculoskeletal problems for many years and went undiagnosed for HPP until middle age. A number of studies concluded that the adult form of HPP is often difficult to diagnose, leading to delayed and sometimes inappropriate treatment [4,11]. Early in 1990, Seshia et al. proposed that clinical symptoms including non-progressive myopathy with muscle pain and stiffness, which were not previously recognized as characteristic HPP, should be considered for diagnosis of milder childhood and adult forms of HPP [38]. More recently, Silva et al. (2012) demonstrated that a non-progressive proximal myopathy with muscle pain and stiffness might represent an early indication of osteomalacia associated with HPP [39]. Likewise, Braunstein et al. (2016) described a 53-year-old man whose main complaints were chronic musculoskeletal pain and fatigue, which were later accompanied by osteomalacia and multiple fractures. In that report, the patient consulted a variety of medical specialists and received several inaccurate diagnoses, including fibromyalgia, costochondritis, thoracic neuritis, chronic pain, plantar fasciitis, and compression arthralgia, and was prescribed pain medications that were ineffective [9]. Low ALP levels and identification of a single heterozygous *ALPL* mutation (c.500C > T, p.Thr167Met) led to the diagnosis of adult-onset HPP.

In the last two decades, case reports and retrospective studies have begun to better recognize and describe less common and/or more generalized symptoms as part of the clinical spectrum of mild forms of HPP [8,13,15,23,40–42]. A common pattern described in these cases includes a healthy childhood and adolescence (although sometimes marked by premature tooth loss that is not followed up or goes unrecognized until years later), with musculoskeletal pain, spontaneous bone fractures, fatigue, and other manifestations beginning in the third, fourth, or fifth decades of life [8–11,41,42]. A natural history study in patients diagnosed with HPP in adulthood by Mori et al. (2016) reported that the majority of patients experienced dental problems in childhood and later in life developed chronic muscle/joint pain and recurrent pathological fractures [11]. Our findings provide another example where long-term follow-up should be emphasized for patients exhibiting premature primary tooth loss associated with low serum ALP levels, as these individuals may encounter other manifestations of HPP in later life.

Dental abnormalities are nearly universal among individuals with HPP, affecting all clinical forms of the disease [14,43,44]. These may include premature primary tooth loss, loss of secondary teeth, tooth mobility, abnormal or thin dentin, large pulp spaces, abnormal root morphology, periodontal disease or alveolar bone loss, malocclusion, and enamel defects. Premature loss of fully rooted primary teeth (particularly incisors) is the most common dental effect of HPP, arising from hypoplasia or aplasia of cementum that causes defective periodontal attachment. This is a hallmark of HPP often useful for diagnosis of childhood, adult, or odonto-HPP forms. In this case, dental observations were key for prompting diagnosis. Increased tooth mobility, abnormal root morphology, and bone loss, coupled with recollection of early primary tooth exfoliation, led to ALP testing and referral to a specialty dental clinic for a comprehensive approach to diagnosis and care. The proband exhibited short rooted permanent teeth, found in upper and lower incisors, canines, pre-molars, and molars. Within the limits of our knowledge, short-rooted permanent teeth have only been reported twice before in HPP patients by Wei et al. [45] and Wang et al. [46]. Of note, our findings did not show visible distinctions regarding the pulp chamber dimension in the proband *versus* the other evaluated family members.

While the mechanisms underlying skeletal and dental mineralization defects in HPP are well described and attributed to accumulation of the TNSALP substrate and mineralization inhibitor, inorganic pyrophosphate (PP_i) [reviewed in 36, 47], the pathophysiology of neuromuscular manifestations such as those described in this family remains poorly understood. Moreover, these manifestations are sometimes poorly correlated to the skeletal findings [reviewed by 12]. Insufficient hydrolysis of PLP (the active form of vitamin B6) is associated with pyridoxine-sensitive seizures, a neurologic dysfunction observed in the most severe cases HPP and correlated with lethality [40]. Loss of TNSALP function causes a central nervous system-localized vitamin B6 deficiency, hyperactivity of central nervous systems and seizures, due reduction of gamma-aminobutyric acid (GABA) or other neurotransmitters [48,49]. Intriguingly, kinetic characterization of TNSALP mutations supports that some mutant forms retained the ability to hydrolyze PP_i, but were inefficient in hydrolyzing PLP, suggesting a mechanism underlying variability in those experiencing seizures [reviewed by 6]. More recently, other functions of TNSALP, including the ability to generate adenosine, a potent inhibitor of nociceptive circuits in the spinal cord that functions in pain signaling and sensory biology, has been recently attributed to 5′ ectonucleotidases such as TNSALP [50]. Reduced hydrolysis of the endogenous pro-nociceptive nucleotides may be a plausible explanation for musculoskeletal pain suffered by individuals with HPP.

It is important to note the limitations of examining a single pedigree and deducing effects of HPP. We cannot say with certainty that all of the signs and symptoms described in the proband and her mother (with milder intensity) were associated with the documented mutations in *ALPL*, and HPP is notoriously variable in its severity even within families, possibly due to the influence of modifier genes [17]. However, evidence from an accumulating number of case reports has begun to

more firmly establish these sorts of musculoskeletal and neurological manifestations with HPP.

5. Conclusion

Within the limits of the present investigation, we conclude that the two *ALPL* mutations identified in this proband and pedigree are associated with neuromuscular, gastrointestinal, and dental manifestations of HPP, further helping to define genotype-phenotype associations for this notoriously variable inherited disorder. This study and others like it will allow further definition of specific sites within the TNSALP molecule potentially related to neuromuscular, gastrointestinal, and other manifestations in adult HPP, providing insights into HPP pathophysiology.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2019.05.005.

Ethics approval and consent to participate

The study protocol was approved by the Piracicaba Dental School - University of Campinas Institutional Review Board (IRB #88910618.0.0000.5418), and the study was conducted in compliance with the recommendation of the World Medical Association Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects. A written informed consent or assent was obtained for all individuals enrolled to report their clinical data, retrospective medical histories, and *ALPL* gene mutations.

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Competing financial interests

The authors declare that they have no conflict of interest.

Authors' contributions

LM contributed to the conception and design of the study, data acquisition and analysis, and manuscript drafting. BF contributed to data analysis and critically revised the manuscript. EJLS, ABA, and KRK contributed to of clinical data acquisition and interpretation, provided oral health care for the patient enrolled in this protocol, and critically revised the manuscript. RDC and RAM contributed to data acquisition and analysis and critically revised the manuscript. FHNJ made substantial contributions to the study's conception and design and critically revised the manuscript. All authors gave final approval of the submitted version and agree to be accountable for all aspects of the work.

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