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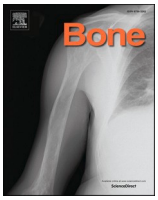

Full Length Article

The Global ALPL gene variant classification project: Dedicated to deciphering variants

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ABSTRACT

Background: Hypophosphatasia (HPP) is an inherited multisystem disorder predominantly affecting the mineralization of bones and teeth. HPP is caused by pathogenic variants in *ALPL*, which encodes tissue non-specific alkaline phosphatase (TNSALP). Variants of uncertain significance (VUS) cause diagnostic delay and uncertainty amongst patients and health care providers.

Results: The *ALPL* gene variant database (https://alplmutationdatabase.jku.at/) is an open-access archive for interpretation of the clinical significance of variants reported in *ALPL*. The database contains coding and non-coding variants, including single nucleotide variants, insertions/deletions and structural variants affecting coding or non-coding sequences of *ALPL*. Each variant in the database is displayed with details explaining the corresponding pathogenicity, and all reported genotypes and phenotypes, including references. In 2021, the *ALPL* gene variant classification project was established to reclassify VUS and continuously assess and update genetic, phenotypic, and functional variant information in the database. For this purpose, the database provides a unique submission system for clinicians, geneticists, genetic counselors, and researchers to submit VUS within *ALPL* for classification. An international, multidisciplinary consortium of HPP experts has been established to reclassify the submitted VUS using a multi-step process adhering to the stringent ACMG/AMP variant classification guidelines. These steps include a clinical phenotype assessment, deep literature research including artificial intelligence technology, molecular genetic assessment, and in-vitro functional testing of variants in a co-transfection model to measure ALP residual activity.

Conclusion: This classification project and the *ALPL* gene variant database will serve the global medical community, widen the genotypic and phenotypic HPP spectrum by reporting and characterizing new *ALPL* variants based on ACMG/AMP criteria and thus facilitate improved genetic counseling and medical decision-making for

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affected patients and families. The project may also serve as a gold standard framework for multidisciplinary collaboration for variant interpretation in other rare diseases.

1. Introduction

Hypophosphatasia (HPP) is a rare, inherited, clinically heterogenous, multisystemic, metabolic disorder [1]. HPP's specific bone and dental defect is impaired mineralization. HPP is caused by loss of function mutations in *ALPL* (NM_000478) located on human chromosome 1p36.12, which encodes tissue nonspecific alkaline phosphatase (TNSALP) [2] and consists of 12 exons covering >70 kb [3]. These pathogenic variants reduce TNSALP activity, leading to the hallmark biochemical signature of decreased serum alkaline phosphatase (ALP), increased serum pyridoxal-5′-phosphate (PLP) and inorganic pyrophosphate (PPi), and increased urinary phosphoethanolamine (PEA).

Diagnosis of HPP is often delayed [4], partly due to phenotypic variability, including a wide spectrum of clinical symptoms. HPP can manifest at any age, and the burden of disease and impact on quality of life can be substantial [5,6]. Clinical symptoms of HPP include defective bone mineralization (radiological, histologic, and clinical features of osteomalacia and rickets), bone deformities, recurrent fractures and pseudofractures, craniosynostosis, neonatal seizures, muscular hypotonia, musculoskeletal pain, failure to thrive, nephrocalcinosis, pseudogout and other extraskeletal calcification, and dental abnormalities with premature exfoliation of teeth and periodontal disease [7–10]. Gene sequencing detects variants in *ALPL*, which to date, is the only gene known to cause HPP. Autosomal recessive and dominant patterns of inheritance are known. However, the results can be inconclusive when variants of unknown significance (VUS) or variants located outside the

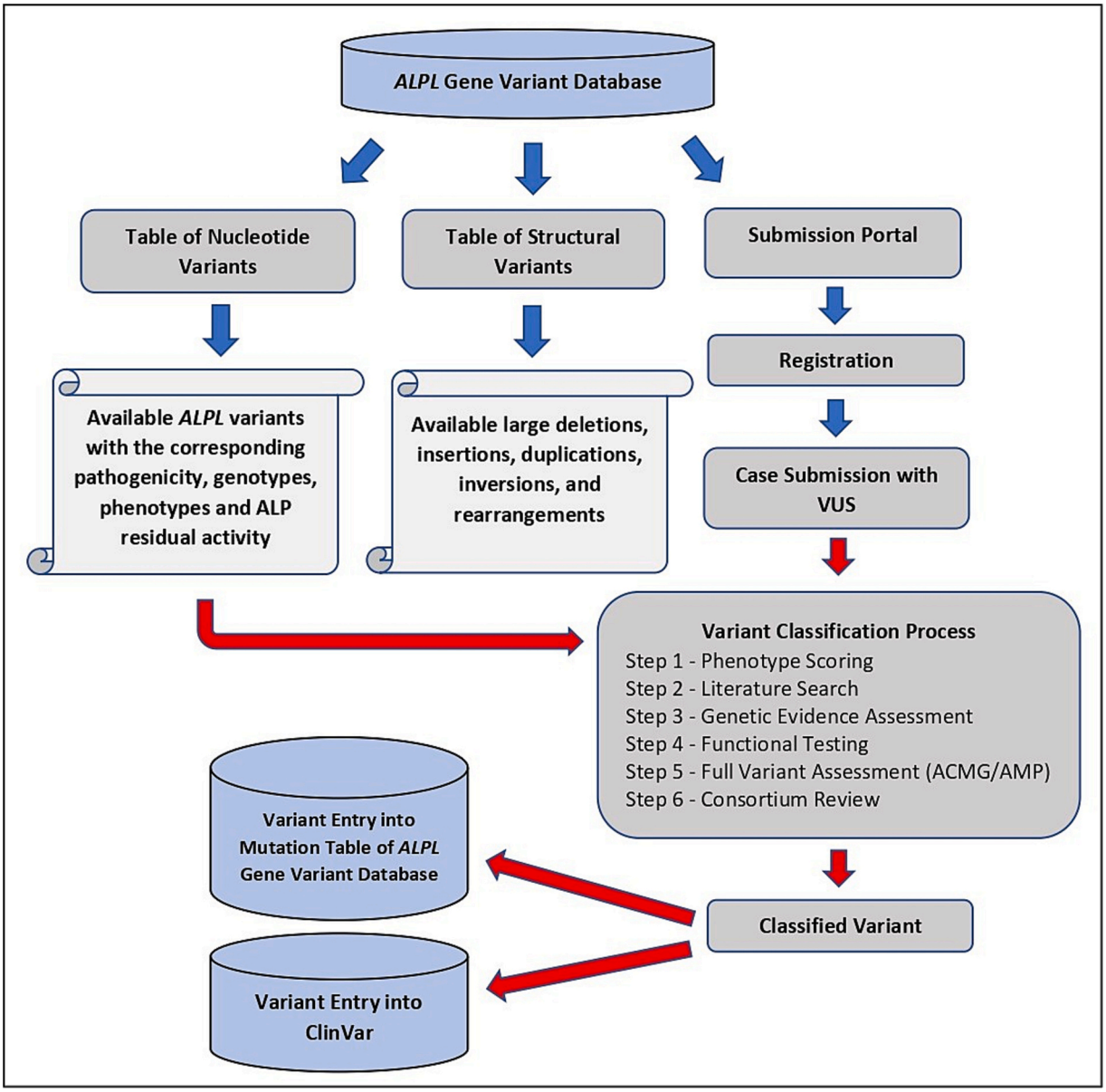

Fig. 1. A flow chart of the *ALPL* gene variant database, the submission portal and classification process. Users can access the table of nucleotide variants on the database's main page, search for variants via a filter and access other functionalities. For submission of VUS, the submission link guides the user through the input of case-specific phenotypic and genotypic data. The blue arrows indicate actions carried out by the user and the red arrows are actions executed by the project staff.

coding regions or splice sites are detected. This may cause uncertainty for patients and treating physicians, prolong diagnostic delay and potentially inappropriate treatment decisions for the patient and affected family members.

Traditionally, clinical HPP phenotypes have been classified into prenatal benign, perinatal lethal, infantile, mild/severe childhood, adult, based on when disease manifestation first occur, and odonto HPP [11,12]. However, the phenotypic spectrum is broad, overlapping and diverse, with variations within and between families. Hence, there is an urgent clinical need for scientific research to better understand the phenotypic and genetic spectrum of the disease. The thorough assessment and reclassification of VUS is an essential step in that regard, not just to define the milder range of the disease, but characterize new and existing HPP phenotypes, all leading to improved certainty and better clinical management.

Therefore, the *ALPL* gene variant project was initiated by scientists at the Johannes Kepler University Linz, Austria. For this purpose, the publicly accessible *ALPL* gene variant database was developed to serve the medical community and an international, multidisciplinary HPP expert consortium was established consisting of clinical HPP experts, experts in genetics and genomics, basic scientists, and biocurators who aim to assess, functionally test and reclassify VUS within *ALPL*.

2. *ALPL* gene variant database

The *ALPL* gene variant database is a freely accessible repository for *ALPL* variants. In April 2020, the SESEP database containing 409 *ALPL* variants, previously curated by Prof. Etienne Mornet, was successfully transferred and redesigned into the new *ALPL* gene variant database available at (https://alplmutationdatabase.jku.at/). The database is designed to provide a comprehensive and easy-to-use tool to store and share information related to *ALPL* variants for the medical and research community caring for HPP. The different functionalities of the database are depicted in Fig. 1.

2.1. Software design and implementation

The *ALPL* gene variant database is a Django Responsive Web application. All the data are stored using PostgreSQL (Version 12.12) on a Linux server (Version 20.04.5 LTS). The website is based on Apache Webserver (Version 2.4.41). The programming languages used are Django Web framework (Python Version 3.8.10), HTML, CSS, JavaScript, and JQuery Library. The queries to the server programs are executed using AJAX technology.

2.2. Database functionalities

2.2.1. Table of nucleotide variants

The nucleotide variant table provides a list of *ALPL* variants, with a filter function as a fast search option. For each variant in the table, the following information is displayed; exon, base change, amino acid change, pathogenicity, reported genotypes, reported phenotypes, residual ALP activity, dominant negative effect, references, type of variant, and links to ClinVar [13] and Mastermind [14] an example shown in Fig. 2. As of October 2023, there are 436 *ALPL* variants in the table and 726 genotypes.

2.2.2. Table of structural variants

This table shows the known large deletions, insertions and duplications, i.e., of whole exons in *ALPL*, inversions and rearrangements. The table also provides the pathogenicity, clinical form, genotype, and references for each structural variant.

2.2.3. The ALPL gene variant classification project

The *ALPL* gene variant database provides a unique feature of a submission portal (https://alplmutationdatabase.jku.at/portal/) for clinicians, geneticists, genetic counselors, and researchers to submit VUS in the *ALPL* gene. Submitted variants then undergo a designated classification process according to criteria set out by the American College of Medical Genetics and Genomics/Association of Molecular Pathology (ACMG/AMP) [15]. The classification process is delivered in 6 steps, as detailed below.

Submission. The submission portal allows the submission of a case with up to 4 variants in *ALPL*. The portal provides forms to be filled in with clinical and phenotypic data of the affected individual and those of other family members carrying the variant, biochemical findings, and signs and symptoms associated with HPP. These facts are then used to assess the likelihood of HPP in this case.

Step 1: Clinical Phenotype Assessment of HPP likelihood. One of the ACMG/AMP criteria (PP4) is based on the phenotypes of the patient and their kindred. Many well-established clinical features of HPP (low serum ALP, elevated PLP/PEA, premature tooth loss with root intact, etc.) have been used for many years in the clinical diagnosis of HPP. However, what is lacking is a formal framework for diagnostic criteria to guide personalized care for individuals based on typical features and severity. To ensure a minimum of phenotypic traits to consider the clinical diagnosis of HPP from submitted or published clinical information, defined diagnostic criteria need to be employed, based upon the clinical, biochemical and radiological features of the disease. Such a framework specifically addresses diagnostic uncertainties at the milder end of the phenotypic spectrum as well as asymptomatic carriers of pathogenic ALPL variants with common age-related conditions such as postmenopausal osteoporosis. Therefore, the consortium lead (WH) first drafted a clinical scoring system designed to correctly identify HPP in individuals of all age groups and all known HPP phenotypes. Vice versa, the scoring system should ideally exclude cases with insufficient clinical evidence. This scoring system was then refined and approved by all consortium members over several months. This weighted scoring system (Table 1) checks for typical and unambiguous signs and symptoms of HPP in current and previous medical history. To consider the clinical

	Location	Base change	Amino acid change	Pathogenicity	Genotypes & Phenotypes	Residual activity	Dominant negative effect	Type of Variant	ClinVar link	Mastermind link
^	Exon4	c.203C>T	p.Thr68Met	Pathogenic	c.[203C>T];[=] Adult [References] c.[203C>T];[529G>A] Childhood [References] c.[203C>T];[571G>A] Childhood [References] c.[203C>T];[814C>T] Perinatal [References]	5.2% Orimo et al. 2001 3.0% del Angel et al. 2020	36.7% del Angel et al. 2020	Missense	Link	Link

Published at
Before November 2019

Amino acid change old nomenclature
Thr51Met

Fig. 2. ALPL gene variant database – table of nucleotide variants. For example, the screenshot for variant c.203C>T demonstrates how the database presents the information. The arrow up/down button on the left hides/reveals additional information.

Table 1
Phenotype scoring sheet (step 1 in variant classification). The sheet depicts how a clinical case is scored for likelihood of HPP. The biochemical signature of HPP carries the largest weight when considering a clinical diagnosis of HPP. The table shows the points allocated to different HPP symptoms, with highest scores for the most unambiguous symptoms or signs in the medical history and data provided. The final score calculated is used to define the strength of the clinical data (PP4) using ACMG/AMP criterion and a phenotype is given to the case.

Phenotypic Scoring Sheet	
PP4 ACMG Strength	**Criteria**
PP4_strong	≥2 cases with ≥3 points in ≥2 genotypes, OR ≥1 case with ≥4 points (not compound heterozygote)
PP4_moderate	≥2 cases with ≥2 points (from different families, not compound heterozygote), OR ≥1 case with ≥ 3 points (not compound heterozygote)
PP4_supporting	≥ 2 points
Proposed Phenotypes (at the time of reporting) – Prenatal benign, perinatal severe, infantile, childhood/juvenile, adult, odonto (symptomatic disease continuum, allowing for new phenotypes) – Asymptomatic with biochemical phenotype (likely benign phenotype 1-2 points) – Asymptomatic without biochemical phenotype (benign phenotype <1 Point)	
Parameter	**Points**
Serum ALP below the lower limit of normal (age- and sex-adjusted) • ALP >50% below the lower limit • ALP <50% below the lower limit	 1.5 1
Elevated serum vitamin B6 (PLP); OR either elevated urine PEA or repeatedly elevated serum phosphate (only if PLP not done)	0.5
X-rays in children (either/or, not additive): • Typical metaphyseal lucency (very specific) • Rickets-like changes on X-ray (flaring, sclerosis, widening)	 2 1
Osteomalacia on bone biopsy	1
Early loss of baby teeth (before age 4y) …with intact root	0.5 1
Chronic musculoskeletal pain (leg/knee/hip)	0.5
Muscular hypotonia or low muscle strength (since birth/young age)	0.5
X-rays/imaging in adults (additive): • Pseudofractures (i.e. atypical femur fractures, or any other location; exclude BP therapy or other conditions) • Any poorly healing fractures, metatarsal fractures • Massive ectopic calcification (after excluding other causes), OR Calcium pyrophosphate deposition (CPPD, pseudogout), pericalcific tendinopathies, ectopic or arterial calcification, current nephrocalcinosis • History of CRMO-like condition/diagnosis	 1 0.5 1 0.5 0.5
Reported age at first symptoms <12 months (incl. failure to thrive, limb deformities, seizures, hypercalcemia or nephrocalcinosis)	0.5
Craniosynostosis	0.5
Death (prenatal - <1y postnatal) from clinically diagnosed severe HPP	3

diagnosis of HPP, the minimum criterion required is the presence of the biochemical HPP signature before considering any other clinical symptoms possibly related to HPP (i.e. neonatal seizures, craniosynostosis, dental, radiological and bony features, muscle hypotonia, musculoskeletal pain, extra-osseous calcifications). Each submitted case is phenotypically scored this way, as well as any additional family members known to carry the variant, if sufficient information is available. The same process is used for cases from the literature search (step 2). For each genotype, the associated phenotypes are determined. In recognition of asymptomatic variant carriers with and without the biochemical HPP signature (low serum ALP, high serum PLP), we added these categories to the list of phenotypes (Table 1). Additional phenotypes could emerge over time, and the scoring system will be adapted accordingly.

Step 2: Literature Search. To support the classification of the variant in question, the responsible biocurator (MF, EBN, EH) conducts an in-depth literature search and extracts reported genotypes containing the variant and associated phenotypes from publications and other databases, i.e., ClinVar and LOVD [16]. In addition, the biocurator also employs artificial intelligence search software (Mastermind; Genomenon Inc., Ann Arbor, MI, USA). The results of the literature search are forwarded to the responsible consortium molecular geneticist for assessment alongside step 1 data.

Step 3: Genetic Evidence Assessment

The responsible consortium molecular geneticist (CR, TM, GW)

reviews all available information submitted and provides a preliminary pathogenicity assessment based on modified ACMG/AMP criteria (Table 2), including population frequency, variant type, protein domain, in silico prediction algorithms (PP3 score), functional and clinical data (PP4 score, from step 1–2) and assessment of the literature. At this stage, the available information may provide sufficient evidence for classification of a variant as pathogenic or benign such that in vitro functional testing may not be necessary. When the functional studies have the potential to upgrade or downgrade a VUS to likely pathogenic/pathogenic or to likely benign/benign based on the in vitro functional test results (PS3) classification criterion, the variant is forwarded to the lab.

Step 4: Functional Testing. The in vitro functional measurement of the variant's residual ALP activity in the research laboratory is designed to assess the pathogenicity of the submitted *ALPL* variant. Twelve controls including four benign and seven pathogenic *ALPL* variants were used to validate the functional testing assay [17]. The functional testing is conducted at our research laboratory at the Johannes Kepler University Linz (JKU) in Linz, Austria, and employs episomal pcDNA3 vectors to create mutant ALP enzyme in ALP-naïve cells [18,19]. Functional testing uses a two-step approach.

The *first and main functional testing method* comprises a co-transfection model expressing mutant and/or wild type *ALPL* in renal MDCK II cells (ECACC, #00062107) and measures residual ALP activity (Fig. 3). To do so, site directed mutagenesis is performed according to the manual instruction (Agilent, #200522) on the ALPL plasmid (OriGene, #RC205692). To confirm whether the correct mutation has occurred through the site-directed mutagenesis, plasmids undergo Sanger Sequencing before introducing them in the co-transfection model. The day before the transfection, cells are seeded at a density of 80,000 cells/cm^2 in 24-well plates and incubated at 37 °C with 5 % of CO2. This density allows to reach 80 % of confluence at the day of transfection. The following day, the medium is replaced with 500 μL of fresh medium and the cells are transiently transfected with either 100 % *ALPL* WT plasmid or 100 % mutant plasmid or 50 % WT / 50 % mutant. In addition, a β-galactosidase plasmid (Promega, #E108A) is co-transfected in every condition to normalize the *ALPL* signal. The transfection is performed according to the manufacturer protocol (Thermo Scientific, #15282465). The ratio DNA/Lipofectamine 3000 used is 1/3 (1 μg DNA plasmids / 3 μL Lipofectamine 3000) and the ratio DNA/P3000 Reagent used is 1/2 (1 μg DNA plasmids / 2 μL P3000 Reagent). Two days after transfection, we analyse ALP and β-galactosidase activities. To do so, cells are lysed using M-PER solution (Thermo Scientific, #11874111) and the lysate is then transferred to a 96-well round bottom dilution plate and centrifuged. Two 50 μL portions of lysate (50 μL β-galactosidase analysis / 50 μL Alkaline Phosphatase analysis) are transferred into a 96-well plate. 50 μl of β-galactosidase reagent (Thermo scientific, #75705) or ALP substrate (obtained by dissolution of 1 tablet *p*-Nitrophenyl Phosphate [PNPP; Thermo scientific, #34047] in 5 mL of Phosphatase Alkaline buffer) is added. Plates are sealed and incubated at 37 °C either for 15 min regarding the ALP treated plate or 2 h regarding the β-galactosidase treated plate. When ALP and PNPP are reacted, a yellow water-soluble reaction product is formed which absorbs light at 405 nm. Thus, the absorbance read at 405 nm for ALP is normalized against that of β-galactosidase at 37 °C. The interpretation of residual ALP activities in this co-transfection model is as follows: A variant is considered "likely pathogenic" (PS3 criterion applied with moderate strength) if residual ALP activity is ≤30 %, and "likely benign" (BS3 applied with moderate strength) if residual ALP activity is >75 %. Of note, the results of 7 known pathogenic controls (11.3–30.4 % mean residual activity range) and 5 known benign controls (71.7–132.4 % mean residual activity range) were consistent with those cut-offs. Although these cut-offs are arbitrary, they are based on extensive previous work by various groups [18,20–23], available information of both severe cases and asymptomatic carriers as well as consortium members' experience.

The *second functional testing method* is only applied (i) if the co-transfection model does not confirm pathogenicity (<30 % residual ALP activity compared to wild type) and (ii) for other types of variants (like intronic/splicing variants, variants in promoter/enhancers) that cannot be functionally analysed by the co-transfection system. Human mesenchymal stem cells (hMSCs) express *ALPL* [24–26]. The variant in question is introduced into immortalized hMSCs using clustered regularly interspaced short palindromic repeats (CRISPR) - associated nuclease Cas9 (CRISPR-Cas9) technology. CRISPR-Cas9 introduced genetic variants in the normal genomic context will use the endogenous cellular transcriptional and splicing machinery. Since CRISPR-Cas9 may induce off-target effects, three different small guide RNAs (sgRNA) and scrambled controls are used. The CRISPR-Cas9 genome editing system requires two components: Cas9, the endonuclease, and a guide RNA (gRNA) which guides Cas9 to a specific location in the genome. With the protospacer-adjacent motif (PAM - the sequence NGG) present at the 3′ end, Cas9 will unwind the DNA duplex and cleave both strands upon gRNA recognition of a target sequence. CRISPR/Cas9 mediated DNA double strand breaks introduced by specific gRNA will target the *ALPL* gene, then the linear donor DNA containing the mutated sequence will be integrated at the gRNA cutting site. The first step includes the generation of immortalized hMSCs stably expressing the Cas9 enzyme. Therefore, we will use viral integration to incorporate a Cas9-expressing plasmid in the cells. The gRNA will then be transiently transfected into the cells. After transfection we will seed single cell clones, and further test them for homozygous tagged ones. If after one round of gRNA/donor DNA transfection, we result in only biallelic clones (one allele has donor integration, the other allele has indels (insertion and deletion) or still contains the wild type sequence), we will repeat the gRNA transfection) using a second target-specific gRNA to obtain homozygous modification of *ALPL*. The mutated clones are Sanger sequenced to ensure the right variants were inserted. Subsequently, the differentiation ability as well as ALP activity will be determined in the genetically engineered hMSCs and compared to scrambled control and parental cells as described in [27].

Step 5: Full variant assessment (ACMG/AMP). The consortium

Table 2
ACMG/AMP criteria for variant classification (step 4 in variant classification). The table organizes the ACMG/AMP criteria by the type of evidence for benign and pathogenic assertion. The evidence code descriptions are BS, benign strong; BP, benign supporting; PM, pathogenic moderate; PP, pathogenic supporting; PS, pathogenic strong; PVS, pathogenic very strong.

Variant Classification			
Strength of criterion	Very strong (PVS1, BA1, BS1-4), Strong (PS1-PS4), Moderate (PM1-PM6), Supporting (PP1-PP5, BP1-BP7)		
Classifications	Benign, Likely benign, VUS, Likely pathogenic, Pathogenic		
Domain	**Data Source**	**ACMG Criteria**	
		Benign	**Pathogenic**
Population frequency	Genome Aggregation Database (exomes and genomes)	BA1, BS1	PM2
Variant type	For example: nonsense, frameshift, consensus splice site, intronic, in-frame indel, synonymous, structural	BP7	PVS1, PM4, PP2
Other substitutions, the same amino acid	Literature		PS1, PM5
Prediction algorithms	Missense (REVEL) Splice (SpliceAI)	BP4	PP3
Functional Domain	Literature		PM1
Clinical data	Submitted phenotype Literature		PP4
Allelic data	Submitted genotype Literature	BP2	PM3
Segregation data	Submitted information Literature	BS4	PP1
Functional studies	Literature *In vitro* functional testing at Johannes Kepler University Linz	BS3	PS3

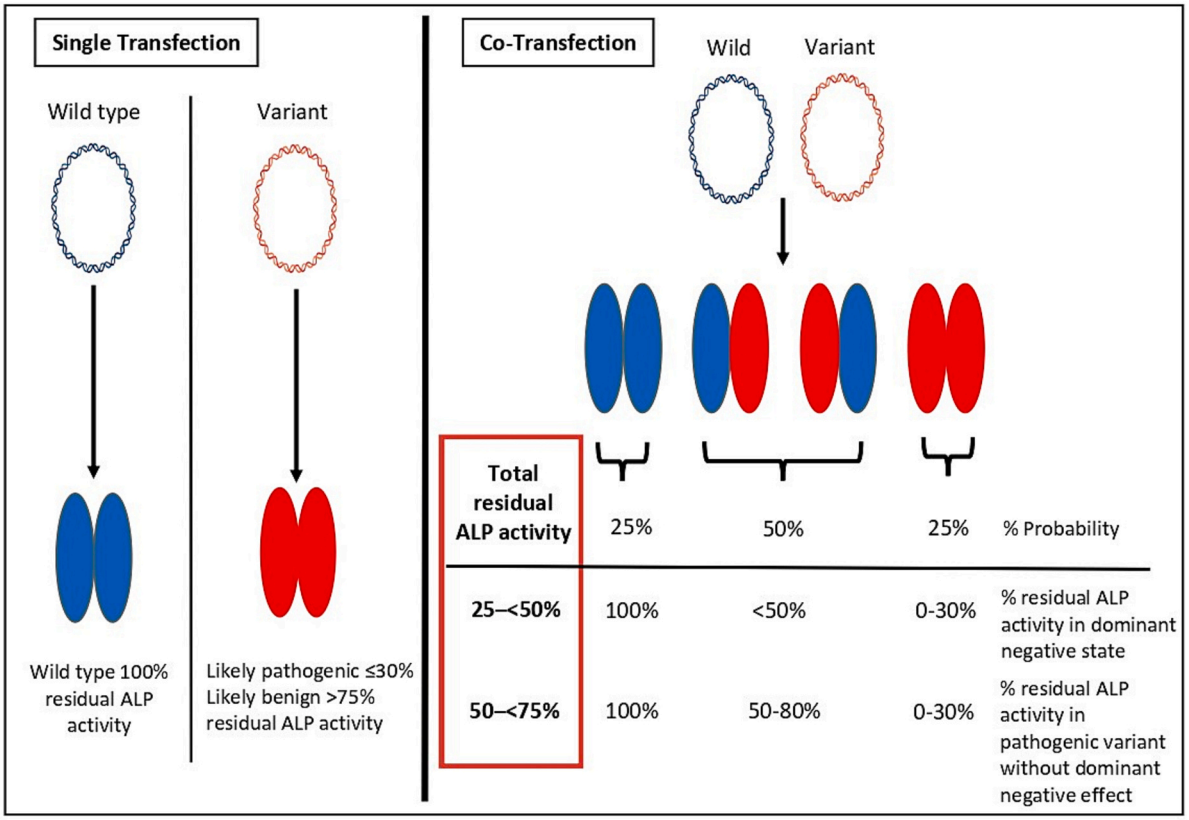

Fig. 3. Predicting in vitro ALP residual activity of a variant. Single transfection of the wildtype *ALPL* (wt only) is taken as 100 % ALP activity. The single transfection of the variant determines its residual ALP activity (relative to wt); if the residual ALP activity is ≤30 % the variant is considered likely pathogenic (PS3 moderate), if >75 % likely benign (BS3 moderate). The co-transfection model determines whether the variant has a dominant negative (DN) effect, which is the case when the total residual ALP activity in the co-transfection experiment is between 25 % and 50 %. This percentage range is derived from probability, given that 25 % of the formed ALP-homodimers are expected to be composed of two wt molecules, 50 % of one wt and one variant molecule, and 25 % of two variant molecules. A DN variant will suppress the wt variant below the expected 50 % total residual activity. However, there will always be 25 % wt homodimers with 100 % ALP activity, so that the expected range is 25–50 % total residual activity. A non-DN variant (last row) represents the heterozygous state, with total residual activities expected to be between 50 and 75 %.

geneticists review all available data for the variant (steps 1–4), including genetic information (i.e., exon, base change, type of mutation, protein domain), in-silico predictions (PP3), clinical score (PP4) based on the phenotypic information submitted through the portal and from the published literature, and in vitro functional test results (PS3). The variant is then classified according to modified ACMG/AMP criteria for *ALPL* (Table 2). The strength of each criterion in the ACMG/AMP classification is categorised as very strong, strong, moderate, and supporting. The consortium geneticists combine criteria according to the ACMG/AMP guidelines and propose a classification of the variant into one of the 5 categories - benign, likely benign, unknown significance, likely pathogenic, and pathogenic.

Step 6: Consortium Review and Variant Classification. The international *ALPL* gene variant consortium consists of a group of 16 members comprising HPP expert clinicians, geneticists, genetic counselors, basic scientists and biocurators from Austria, Canada, France, Germany, Spain, Japan, and the USA. Consortium members are presented with the summary details of all data sources and debate the collected data on each variant and the classification proposal. The consortium members who attend monthly teleconferences to classify *ALPL* variants may ask for further information, or otherwise vote with simple majority to accept or reject the classification proposal. All information that leads to the classification is saved in an electronic file and stored for future reference, with all ACMG/AMP criteria used and date of consortium decision. The successfully classified variant is then added to the table of variants in the JKU database.

Submission to ClinVar. The newly classified variants are also submitted to ClinVar.

3. Discussion

When exploring the genotypic spectrum of any rare disease, it is essential to uphold ACMG/AMP rules for variant classification, as this process – in the absence of firm criteria for the clinical diagnosis of HPP - indirectly affects clinical diagnosis making. With expanding knowledge on HPP phenotypes and associated *ALPL* variants, diagnostic criteria and thus the indications for considering enzyme replacement therapy [28,29] need to be sharpened, which highlights a growing need to reach consensus internationally on how exactly the diagnosis of HPP is made. This particularly applies to the milder and more attenuated end of the clinical spectrum, specifically because joint or musculoskeletal pain and weakness and low-turnover conditions (with low ALP) are common in adults. In addition, low ALP, as the biochemical hallmark feature of HPP, is not specific to HPP, but a common finding in many conditions and situations [18,30]. The phenotype scoring employed in this project represents only one piece of evidence (PP4) that joins other domains in establishing variant pathogenicity by ACMG/AMP criteria. As such, no phenotypic score is “diagnostic” or “exclusive” in its own right. A true odonto HPP patient would only ever score a maximum of 2 (biochemical phenotype max 1.5 + dental 0.5). A score lower than 2 makes HPP unlikely, a score above 2 more likely. The scoring is taken with great caution in awareness of other bone conditions that may mimic phenotypic features, will be continuously refined and will be validated in the future once the wider phenotypic spectrum of HPP is uncovered and

clearer. The *ALPL* gene variant project, with its stringent steps and well-defined classification process, will provide the evidence to improve diagnostic certainty for clinicians, geneticists and, foremost, affected families.

3.1. Comparison with information in other databases

Other freely accessible databases, i.e., ClinVar and LOVD are archives of human genetic variants and interpretations of their significance for reported conditions. Although these databases provide partly similar information on *ALPL* variants as the *ALPL* gene variant database, i.e., cDNA code, protein code, clinical information, and references to publications, the *ALPL* gene variant database additionally provides the reported genotypes for each variant and the corresponding phenotypes for each genotype, which is missing from other databases. One feature of the *ALPL* gene variant database that stands out is the stringent variant classification process based on well-defined criteria and functional testing, that other databases do not provide.

3.2. New phenotypes emerging

The phenotype of HPP is known to be variable; in particular, individuals in the more attenuated spectrum of the condition provide a diagnostic challenge. The historically described phenotypes by disease onset (prenatal benign, perinatal severe, infantile, childhood, adult, odonto) don't fully reflect an overlapping symptomatology, the evolving nature of the disease and the full disease spectrum. In addition, even siblings with identical *ALPL* genotypes may have different HPP phenotypes [31]. Through studying the submitted and reported phenotypes, we already discovered two different types of asymptomatic heterozygote "carriers" – those with and without typical biochemical features of HPP, and refined Table 1 accordingly. Asymptomatic carriers with a biochemical signature have been discovered in laboratory screening studies [32–34]. However, the definition and terminology "asymptomatic carriers" may be subject to reporting bias, since the current spectrum of symptoms only reflects what is historically understood as an HPP-related clinical manifestation. This limitation actually applies on two levels. Firstly, submitters may only report symptoms they perceive to be associated with the disease and secondly, the parameter list applied to assess the clinical phenotype (Table 1) is always restricted to what is current state of the art knowledge. Therefore, we specifically allow the submission of additional important clinical information, and that of family members, to ensure recognition of new or alternative phenotypes.

Until further evidence emerges, clinicians should be aware of biochemical disease signatures present in supposedly asymptomatic people who should not be diagnosed with clinical HPP (avoid overdiagnosis), but still monitor such individuals with a sole biochemical phenotype for potentially emerging, or as yet unrecognized, symptoms or signs. In our continuous effort to elucidate this condition, the *ALPL* gene variant consortium will strive to better describe these phenotypes over time and encourage the international community to contact us, not just with new genotypes but also new phenotypes or phenotypic patterns.

4. Future directions

To keep the *ALPL* gene variant database up to date and complete, we continuously screen the new scientific literature and enter new variants, genotypes and phenotypes reported by other groups into our database. In addition, the consortium will continuously develop and validate the phenotype scoring (Table 1) and revisit the arbitrary cut-offs for ALP residual activity employed in functional testing. To help us achieve a comprehensive, complete database for *ALPL* variants we encourage clinicians, geneticists and genetic counselors to submit new variants via our submission portal and also report new genotypes or phenotypes for existing/known variants in the ALPL gene variant database to us at hpprresearch@jku.at.

We recognize that the current laboratory functional testing methods (Step 4) are only applicable for missense variants (co-transfection), and intronic/splicing and promoter/enhancer variants (CRISPR-Cas9). More complex structural variants cannot be functionally assessed this way, so their classification is limited to relying on prediction programs, although other methods based on RNA evaluation are being considered. In addition, historically, various methods and substrates have been used for testing residual ALP activity in-vitro, with varying sensitivity and specificity, so results are not necessarily comparable. There is also emerging data indicating that the TNSALP expression level, more than solely residual ALP activity, may determine pathogenicity [35]. In addition, all elements involved in ACMG/AMP classification applied in this project are subject to continuous adaptation to emerging evidence. Future updates of the ACMG/AMP classification system will also mandate revisions to our classification system.

5. Conclusion

The *ALPL* gene variant database will provide a powerful tool for clinicians and scientists to look up variants and view evidence on their pathogenicity. The number of listed variants and quality of information will grow continuously through the consortium's reclassification activity, submission of variants and incorporation of variants from literature research. Through the work of the consortium, the *ALPL* gene variant project will contribute substantially to the diagnosis and clinical management of HPP.

Accessibility and usage

The database is publicly accessible for search through the *ALPL* gene variant database URL (https://alplmutationdatabase.jku.at/). To submit new variants (VUS), access the submission portal through the URL (https://alplmutationdatabase.jku.at/portal/), simply click on the 'Submission' button located on the main page. All the necessary submission guidelines can be found there. To report new genotypes or phenotypes of existing variants in the database, contact the curator by email at hpprresearch@jku.at.

Citation

Publications arising from the use of the *ALPL* gene variant database should cite this article and the accompanying URL (https://alplmutationdatabase.jku.at/).

CRediT authorship contribution statement

Mariam R. Farman: Writing – review & editing, Writing – original draft, Investigation. **Catherine Rehder:** Writing – review & editing, Methodology, Investigation. **Theodora Malli:** Writing – review & editing, Investigation, Data curation. **Cheryl Rockman-Greenberg:** Writing – review & editing, Resources, Data curation. **Kathryn Dahir:** Writing – review & editing, Resources. **Gabriel Ángel Martos-Moreno:** Writing – review & editing, Data curation. **Agnès Linglart:** Writing – review & editing, Supervision. **Keiichi Ozono:** Writing – review & editing, Supervision, Data curation. **Lothar Seefried:** Writing – review & editing, Supervision, Data curation. **Guillermo del Angel:** Writing – review & editing, Methodology. **Gerald Webersinke:** Writing – review & editing, Supervision, Methodology, Data curation. **Francesca Barbazza:** Writing – review & editing, Methodology. **Lisa K. John:** Writing – review & editing, Methodology. **Sewmi M.A. Delana Mudiyanselage:** Writing – review & editing, Methodology. **Florian Högler:** Writing – review & editing, Investigation, Data curation. **Erica Burner Nading:** Writing – review & editing, Methodology, Data curation. **Erin Huggins:** Writing – review & editing, Methodology, Data curation. **Eric T. Rush:** Writing –

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Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

Not applicable for that section.

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Authors contributions

WH, AEG and MRF contributed in the design of the *ALPL* database and coordination of this project. MRF and WH were the major contributors to the writing of the manuscript. MRF and FH performed literature search, collected data and prepared *ALPL* variants for reclassification. WH analysed the clinical data. AEG, FB, LJ, SDM performed lab experiments for functional testing of *ALPL* variants. CR, TM, GW, EN and EH performed genetic assessment and reclassified the *ALPL* variants. All consortium members attend monthly consortium meetings and agree on the reclassification of variants. All authors reviewed and provided input on the manuscript. All authors read and approved the final manuscript.

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CRG, KD, GAMM, AL, KO, LS, PSK, WH are steering committee members of the HPP global registry sponsored by Alexion, AstraZeneca Rare Disease, have worked as consultants, and received research funding and honoraria from Alexion, AstraZeneca Rare Disease. PK is a member of the Scientific Advisory Board for Alexion Pharmaceuticals, Inc. ETR has worked as a speaker, consultant, and has received research funding from Alexion AstraZeneca Rare Disease. GdA is a current employee of Alexion, AstraZeneca Rare Disease and may own stock and/or options therein.

Consent for publication

Not applicable. The manuscript does not contain any individual person's data in any form. All authors agree to publishing this manuscript.

Ethics approval

The project has been approved by the Ethics Committee of the Medical Faculty of JKU, Linz, Austria (EK Nr: 1118/2021). Submitting geneticists and physicians request approval by their local IRBs unless waivers are in place. Patient information and consent forms can be downloaded from the project website. https://alplmutationdatabase.jku.at/portal/

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