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Mild hypophosphatasia may be twice as prevalent as previously estimated: an effective clinical algorithm to detect undiagnosed cases

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Abstract

Objectives: Since the prevalence of hypophosphatasia (HPP), a rare genetic disease, seems to be underestimated in clinical practice, in this study, a new diagnostic algorithm to identify missed cases of HPP was developed and implemented.
Methods: Analytical determinations recorded in the Clinical Analysis Unit of the Hospital Universitario Clínico San Cecilio in the period June 2018 – December 2020 were reviewed. A new clinical algorithm to detect HPP-misdiagnosed cases was used including the following steps: confirmation of persistent hypophosphatasemia, exclusion of secondary causes of hypophosphatasemia, determination of serum pyridoxal-5′-phosphate (PLP) and genetic study of *ALPL* gene.
Results: Twenty-four subjects were selected to participate in the study and genetic testing was carried out in 20 of them following clinical algorithm criteria. Eighty percent of patients was misdiagnosed with HPP following the current standard clinical practice. Extrapolating these results to the current Spanish population means that there could be up to 27,177 cases of undiagnosed HPP in Spain. In addition, we found a substantial proportion of HPP patients affected by other comorbidities, such as autoimmune diseases (~40 %).
Conclusions: This new algorithm was effective in detecting previously undiagnosed cases of HPP, which appears to be twice as prevalent as previously estimated for the European population. In the near future, our algorithm could be globally applied routinely in clinical practice to minimize the underdiagnosis of HPP. Additionally, some relevant findings, such as the high prevalence of autoimmune diseases in HPP-affected patients, should be investigated to better characterize this disorder.

Trinidad González-Cejudo and Juan Miguel Villa-Suárez contributed equally to this work.

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Keywords: diagnostic algorithm; hypophosphatasia; prevalence; rare diseases

Introduction

Hypophosphatasia (HPP) is a rare genetic disease caused by a loss-of-function mutation in the gene encoding tissue-nonspecific alkaline phosphatase (TNSALP) [1–3]. TNSALP is involved in the dephosphorylation of a wide range of substrates, including inorganic pyrophosphate (PPi), phosphatidylethanolamine (PEA), pyridoxal-5′-phosphate (PLP) [4–7], and others such as adenosine triphosphate (ATP), di-phosphoryl lipopolysaccharide (LPS), and phosphorylated osteopontin (p-OPN) [8–11]. HPP is considered an innate error of metabolism with highly variable clinical presentation, and HPP is usually involved in bone and tooth mineralization abnormalities [11, 12].

Previous studies have shown that the severity of the disease is inversely related to the age at which symptoms appear (except for benign HPP). Thus, perinatal lethal and infantile HPP are the most severe forms, while affected adults have milder HPP forms, with premature tooth loss (odontohypophosphatasia) [13] and increased risk of fractures, chronic pain and joint inflammation, including, in some cases, neurological disorders [14–19]. Although the main HPP-related symptomology is bone deterioration, this disorder shows great variability in its clinical presentations due to its great genetic heterogeneity (more than 500 mutations have been described in the *ALPL* gene to date) [20, 21]. In this context, there is increasing evidence of patients with symptoms other than the typical pathognomonic signs of HPP, so this disease is being increasingly considered a multisystemic disease [9, 17, 22, 23].

In terms of prevalence, HPP is currently classified as an ultrarare disease since several studies describe low rates of affected individuals around the world [2, 14, 24]. However, studies previously conducted by García-Fontana et al. and Mornet et al. [12, 22] have shown that HPP may have a higher prevalence than originally estimated, being underdiagnosed in clinical practice due to the overlap of its clinical symptoms with those of other more prevalent diseases, such as osteoporosis or osteomalacia [25, 26]. This fact has important consequences since the use of some antiosteoporotic drugs in HPP patients, such as bisphosphonates that remain for a long time in the body, worsens the prognosis of the disease [18, 27–29].

Currently, low ALP levels are not considered to be a relevant symptom of any major disorder, so HPP often remains unrecognized. In this context, the clinical laboratory plays a crucial role in the diagnosis of HPP to avoid the underdiagnosis of this disorder. Thus, the reporting of decreased values below normal limits for ALP is a recent update to clinical practice at University Hospital Clínico San Cecilio of Granada.

The recent availability of an effective HPP treatment based on the enzymatic replacement of ALP has generated the need to establish better diagnostic algorithms for early detection of the infant-juvenile form, as well as to re-evaluate those patients with high clinical suspicion of HPP who may have been misdiagnosed with other diseases.

The proactive search for this disorder by means of a structured and systematized analysis will make it possible to know the real prevalence of HPP in each population and to establish appropriate preventive and therapeutic measures.

In this context, the aim of this study was to design and assess the usefulness of an effective protocol to improve the diagnosis of HPP that can be implemented in clinical practice for the early and reliable detection of HPP. In addition, we intended to further investigate the extraskeletal effects of low ALP levels in the affected population to better characterize this disease.

Materials and methods

Study population

To carry out this study, all the clinical analysis records of subjects with any determination of serum ALP levels from 1 June 2018 – 31 December 2020 at the Clinical Analysis Unit of the University Hospital Clínico San Cecilio in Granada were reviewed. After reviewing the clinical analysis records, 64,013 patients (62,412 adult subjects and 1,601 pediatric subjects) were selected for the evaluation of the new diagnostic algorithm.

Patients included in the study had persistently decreased ALP activity (at least in two assessments) considering the reference ranges based on the CALIPER study [30] for the pediatric population and proposed by the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) [31] for the adult population (Table 1).

Patients with any condition that had previously been identified as a secondary hypophosphatemia cause (coronary artery bypass surgery, diseases that could decrease ALP levels, treatment with potent bone antiresorptive agents, or massive transfusion) or those whose blood had been collected incorrectly (in the presence of oxalate or absence of ethylene diamine tetra acetic acid (EDTA)) were excluded [32–36].

Selected subjects completed a quality-of-life questionnaire, a project-specific informed consent document and an additional informed consent document for the HPP genetic study, performed at the Biomedical Diagnostic Centre of the Hospital Clinic of Barcelona.

Two blood samples were drawn from each patient and stored at −80 °C to determine PLP levels (from plasma sample) and to perform the sequencing of coding regions of the *ALPL* gene and flanking regions (from whole blood sample).

The present study was approved by the Granada provincial Research Ethics Committee (CEI Granada) (ID Project: 0777-M1-20) on 8

Table 1: ALP reference values stratified by age and sex based on the CALIPER study and the IFCC standards for the pediatric and adult populations, respectively.

Sex	Age	L, U/L	H, U/L	Reference
Female	0–14 days	76	233	CALIPER
Female	15 days–1 year	113	443	CALIPER
Female	2–9 years	132	315	CALIPER
Female	10–12 years	119	393	CALIPER
Female	13–14 years	52	239	CALIPER
Female	15–16 years	45	108	CALIPER
Female	17–18 years	40	80	CALIPER
Female	>18 years	33	98	IFCC
Male	0–14 days	76	233	CALIPER
Male	15 days–1 year	113	443	CALIPER
Male	2–9 years	132	315	CALIPER
Male	10–12 years	119	393	CALIPER
Male	13–14 years	107	442	CALIPER
Male	15–16 years	75	312	CALIPER
Male	17–18 years	49	139	CALIPER
Male	>18 years	43	115	IFCC

L, lower value; H, higher value.

May 2019 and was performed in accordance with the principles of the Declaration of Helsinki of the World Medical Association.

Diagnostic algorithm to identify potential HPP cases

A diagnostic algorithm was designed and implemented in the Clinical Analysis Unit of the University Hospital Clínico San Cecilio of Granada for the early detection of HPP.

The HPP diagnostic protocol starts through the laboratory computer system database, identifying ALP assessments below the reference values based on the reference ranges shown in Table 1.

When there are two or more ALP results in the medical record and one of them remains near the reference values, the ALP levels are analyzed in future analyses.

If there is only one determination of serum ALP levels in the medical record, and the level is below the reference values, a new determination is requested to check whether the decreased ALP levels are persistently maintained.

In the case of persistently low ALP values, secondary causes of hypophosphatemia [32–36] are ruled out. After this, the patients with suspected HPP are referred to the pediatric or endocrinology unit, where the patients are informed about HPP disease and the procedure to be followed. Two blood samples are collected from participants to determine their serum PLP levels and to perform Sanger sequencing of the *ALPL* gene if PLP levels are above the reference values (3.6–18 ng/mL) to identify possible genetic variants associated with HPP (Figure 1).

Biochemical parameters

Measurements of ALP activity in serum samples were performed on an AU5800 analyzer (Beckman Coulter, California, USA) by absorbance spectrophotometry. The ALP activity was determined by measuring the rate of the conversion of p-nitrophenyl phosphate (pNPP) to p-nitrophenol (pNP) in the presence of magnesium and zinc ions and of 2-amino-2-methyl-1-propanol (AMP) as a phosphate acceptor at pH 10.4. The rate of change in absorbance due to the formation of pNP was measured bichromatically at 410/480 nm, with this rate being a direct function of the ALP activity in the sample. A less than 5 % coefficient of variation (CV) was determined for the run precision of the assay, and a less than 10 % CV was determined for the overall precision. Regarding sensitivity, the typical absorbance change per minute for 1 U/L ALP was 0.22 mA absorbance.

PLP concentrations in plasma were determined using EDTA3K tubes at the Clinical Analysis Unit of the Niño Jesús Pediatric University Hospital (Madrid, Spain) by high-performance liquid chromatography (HPLC) (Agilent 1200 Series isocratic HPLC system) with a fluorescence detector (Agilent G1321A 1200 Series) using the sample preparation kit from Chromsystems Instruments & Chemicals GmbH according to the manufacturer's recommendations. The excitation and emission wavelengths of the detector were 320 and 415 nm, respectively. The injection volume was 50 µL with a flow rate of 1.2 mL/min, with a precision of <0.5 % relative standard deviation (RSD) over an injection volume range of 0.01–2000 µL. The fluorescence detector has a data rate of 74 Hz for spectral mode, showing low limits of detection with single-wavelength Raman (H_2O)>500 and dual-wavelength Raman (H_2O)>300. Reference PLP values ranged from 3.6 to 18 ng/mL.

Genetic analyses

Whole blood samples were processed by the Biomedical Diagnostic Centre of the Hospital Clinic of Barcelona, where DNA isolation and *ALPL* gene sequencing were performed. Genomic DNA was extracted from peripheral blood lymphocytes using the MagNa Pure 96 DNA and Viral NA Large Volume Kit in the automated DNA extractor MagNa Pure 96 System (Roche Life Science, Switzerland) according to the manufacturer's instructions, and the *ALPL* gene was amplified by PCR. Subsequently, Sanger sequencing was performed using the PCR product as a basis and the truncated sequence NM_000478.5 as a reference to determine the sequence of the coding regions and exon–intron junctions of the *ALPL* gene. Next, a study of copy number variants was performed by multiplex ligation probe amplification (MLPA) (MRCHolland). The results were analyzed using the SeqPilot program (JSI Medical Systems).

Results

Results of the application of the diagnostic algorithm

Initially, 64,013 patients with ALP determinations in the 30-month period from 1 June 2018 to 31 December 2020 were selected (62,412 subjects in the adult population and 1,601 subjects in the pediatric population). Of these, a total of 765 subjects with decreased ALP levels were selected following the diagnostic algorithm described previously. Among them, 522 subjects were excluded because they had only a single ALP determination. Of the resulting 243 subjects (204 in the adult population and 39 in the pediatric population), 128

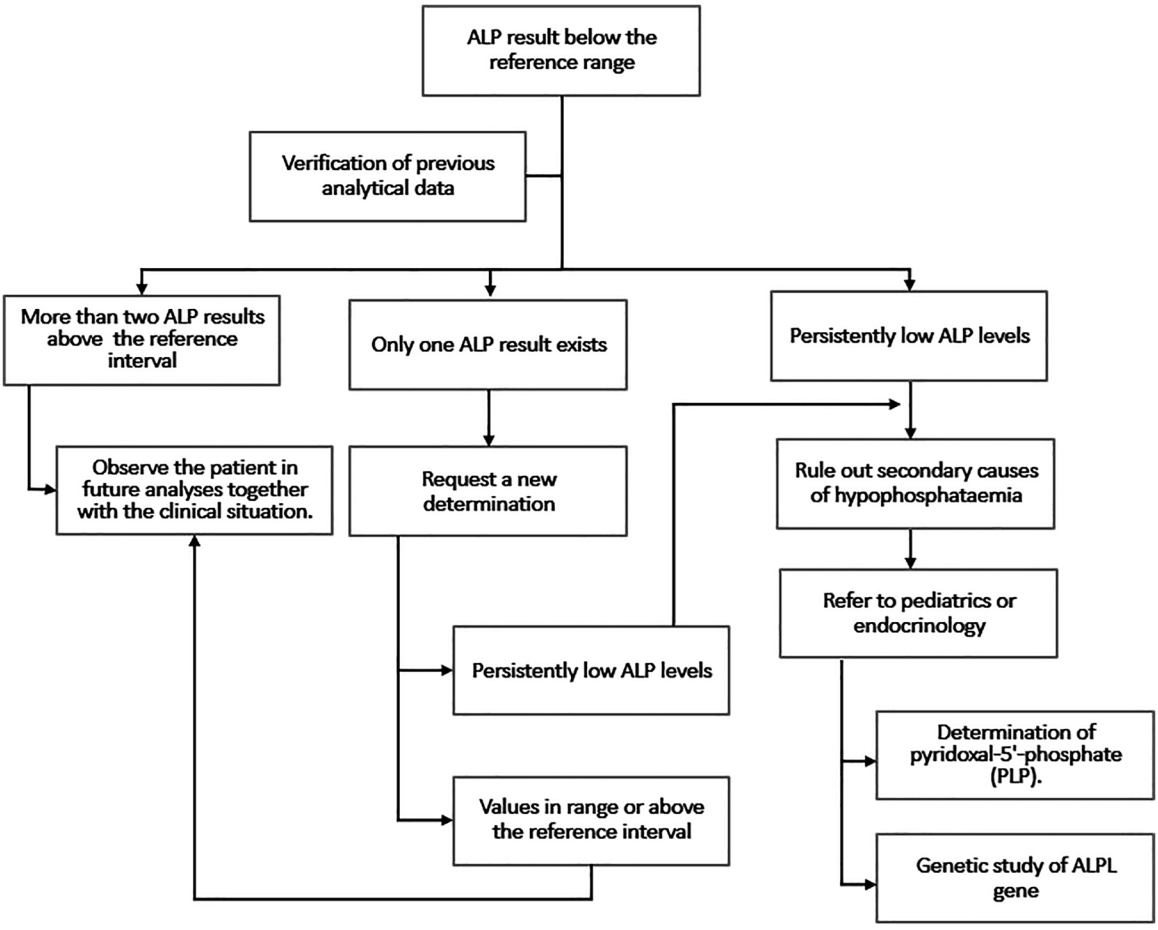

Figure 1: Diagnostic algorithm to identify potential HPP cases.

were excluded for having two or more ALP values in or above the reference range. After studying the medical records of the remaining 115 patients, 54 subjects were excluded for having possible causes of secondary HPP. Finally, only 24 of the 61 subjects with suspected HPP participated in the study (21 adult subjects and three pediatric subjects) (Figure 2).

The ALP and PLP levels of the 24 patients who agreed to participate in the study were determined. Analyzing the values observed in the adult population (n=21), we observed means of 24.8 (±7.1) U/L and 91.6 (±106.0) ng/mL, for ALP and PLP respectively. Considering all participating subjects (n=24), 17 adults and three children had persistently decreased ALP activity and increased PLP levels compared to baseline values. These 20 patients underwent genetic screening.

Genetic results

Mutations in the *ALPL* gene were identified in 16 of 24 participating subjects (14 adults and two children) (Tables 2 and 3). This means that 67 % of the participating subjects were affected by HPP. However, considering the patients who underwent genetic testing (20 subjects), the proportion of patients affected by HPP increased to 80 % when our diagnostic algorithm was applied.

All of the mutations found in the *ALPL* gene were heterozygous mutations. We identified mutations previously described as pathogenic mutations in eight adult subjects (patients 4, 6, 13, 14, 16, 17, 18 and 20; Table 2) and mutations previously described as likely pathogenic mutations in three adult subjects (patients 10, 11 and 19; Table 2). Additionally, mutations of uncertain significance were found in three subjects (patients 1, 15 and 21; Table 2).

Additionally, regarding the pediatric subjects, we found two patients (i.e., almost 67 % of the pediatric population [patients 2 and 3; Table 3]) with heterozygous mutations in the *ALPL* gene not previously described in the scientific literature.

Biochemical and clinical features

Most of the subjects who participated in the study (20/24 individuals; 83.3 %) presented pathognomonic symptoms of

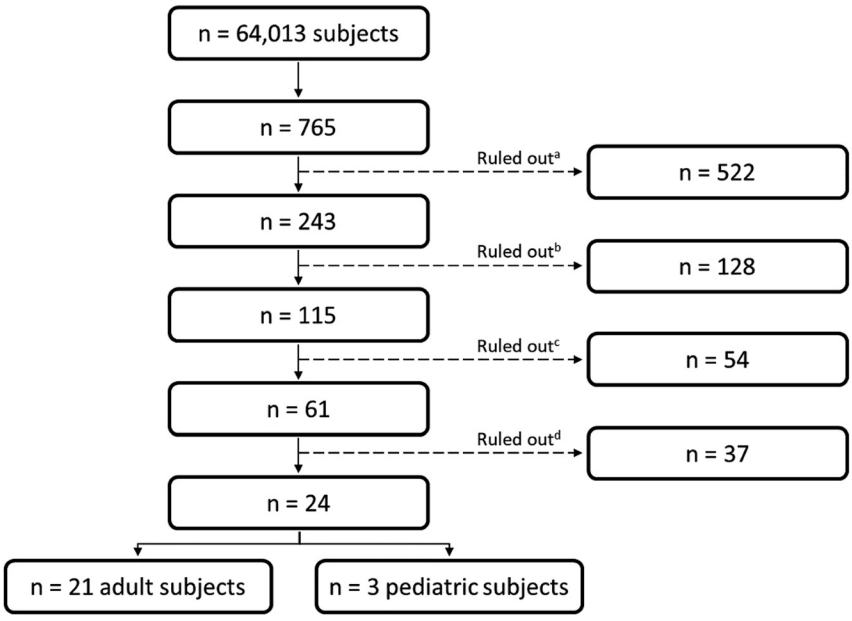

Figure 2: Flowchart of the recruitment of patients potentially affected by HPP. (a) Excluded for having only a single ALP determination. (b) Excluded for having two or more ALP values in or above the reference range. (c) Excluded for having possible causes of secondary hypophosphatasia. (d) Excluded for refusing to participate in the study.

HPP. In fact, one of them had already shown rheumatologic symptoms in his childhood (patient 19, Table 2), so this could be a case of undiagnosed infantile HPP. Only two patients had a history of fractures, one of them with a fracture of the left shoulder trochlea (patient 14, Table 2) and the other with a fracture of the left carpal scaphoid (patient 18, Table 2). The latter patient had also suffered several losses of teeth with complete root fractures.

In addition, approximately 4 % of the patients (patient 6, Table 2) presented recurrent nephrolithiasis. Furthermore, none of the patients had a family history of HPP or rickets in childhood.

However, four patients had no HPP-related symptomatology (patients 4, 8 and 11 (Table 2) and patient 3 (Table 3)). Notably, all these patients except for patient 8 also presented biochemical and genetic features compatible with the diagnosis of HPP, so these could be asymptomatic cases of HPP.

Finally, it is interesting to highlight that 6/16 subjects with a mutation in the *ALPL* gene (37.5 %) presented some autoimmune conditions, as these disorders are potential comorbidities not associated with HPP to date.

Discussion

Since HPP is a little-known disease and low serum ALP levels are not usually considered in clinical practice as indicators of major disorders, HPP is often underdiagnosed, as we have suggested in previous studies [22]. In this study, a new HPP detection algorithm was proposed by determining low ALP levels using the values stratified by age and sex as established by the standards of the CALIPER study for the pediatric population and the IFCC study for the adult population.

After clinical characterization and genetic testing of the patients, 16 of the 24 study participants (14 adults and two pediatric individuals) showed a mutation in the *ALPL* gene. According to these data, more than half of the studied population (66.67 %) was not correctly diagnosed following standard clinical practice. If we extrapolate these data to the current population of Spain, this means that 10,779 potential cases of HPP exist that are not currently diagnosed. Considering the proportion of potential HPP patients who did not participate in this study for different reasons (60 %) and the proportion of patients with positive genetic results in the present study, the current estimate of potential undiagnosed HPP cases in Spain could be as high as 27,177 cases, which means that the current HPP prevalence could be 1/1,692 for mild forms. In this context, in 2011, Mornet et al. estimated a mild HPP prevalence of 1/6,370 for Europe [14]. In our previous study in 2019, we estimated a mild HPP prevalence for Spain that duplicated the European mild HPP prevalence estimated by Mornet (1/3,100) [22], which was similar to the new estimation calculated by Mornet et al. in 2021 for Europe (1/2,430) [12]. Thus, in this study, using our

Table 2: Clinical characteristics, ALP levels, PLP levels and genetic testing results of the adult population.

Patient	Age	Sex	Bone fractures	Dental abnormalities	Main diagnosis	ALP (M: 43–115 U/L; F: 33–98 U/L)	PLP (3.6–18 ng/mL)	Genetic testing results	Location
1	62	M	No	No	Gonalgia, meniscopathy, benign prostatic hypertrophy	16	[a]119	c.875C>T; p.(Pro292Leu)	Exon 9
2	60	M	No	No	Acromegaly, benign prostatic hypertrophy, lumbosarthrosis, calcifying tendinitis of the shoulder	25	[a]61	(–)	–
3	61	M	No	No	Right knee patellofemoral arthrosis, hemochromatosis	32	[a]50	(–)	–
4	32	M	No	No	Pulmonary valve stenosis	24	[a]145	c.382G>A; p.(Val128Met)	Exon 5
5	69	M	No	No	Osteoporosis, polyarthralgia, rheumatoid arthritis[b], spurs	35	11.15	Does not apply	–
6	50	M	No	No	Scheuermann's kyphosis, renal lithiasis, femoral enchondroma, nonspecific arthralgias, osteoporosis	35	[a]20.3	c.571G>A; p.(Glu191Lys)	Exon 6
7	24	M	Yes	No	Clavicle fracture, femur amputation (car accident)	26	14.6	Does not apply	–
8	64	M	No	No	Biliary stenosis, chronic renal failure	20	9.13	Does not apply	–
9	53	M	No	No	Osteopenia, dermatomyositis[b], hemochromatosis	34	[a]19.1	(–)	–
10	33	M	No	No	Psoriatic spondyloarthrosis[b], left gonalgia, low back pain, scoliosis, immune thrombocytopenic purpura[b], shoulder and left foot pain	27	[a]120	c.1327G>A; p.(Ala443Thr)	Exon 12
11	64	M	No	No	Coronary heart disease, type 2 diabetes mellitus	27	[a]122	c.1366G>A; p.(Gly456Arg)	Exon 12
12	56	M	No	No	Lumboarthrosis, bilateral acromioclavicular arthrosis, coxarthrosis, clavicle arthralgia, polycythemia vera, psoriasis[b]	28	11.62	Does not apply	–
13	46	F	No	No	Lupus[b], joint pain, distal metatarsalgia	21	[a]33.2	c.473-2A>G	Intron 5
14	63	F	Yes	No	Polyarthritis[b]; osteochondritis; rickets; gonarthrosis; synovitis, acne, pustulosis, hyperostosis, osteitis (SAPHO) syndrome[b]	21	[a]28.6	c.473-2A>G	Intron 5
15	27	F	No	No	Knee and lower back pain, goiter, autoimmune thyroiditis[b]	23	[a]34	c.69_74del; p.(Glu23_Lys24del)	Exon 3
16	41	F	No	No	Lumbar facet syndrome, gonarthrosis grade I, polyarthralgia, supra-spinatus tendinopathy	30	[a]43	c.558G>A; p.(Trp186[a])	Exon 6
17	61	F	No	No	Osteoporosis, spondyloarthrosis with osteophytosis	15	[a]160	c.571G>A; p.(Glu191Lys)	Exon 6
18	40	F	Yes	Yes	Polyarthralgia, dorsal scoliosis, sternocostal osteochondritis	30	[a]59	c.407G>A; p.(Arg136His)	Exon 5
19	29	F	No	No	Gonalgia, gonarthrosis, osteochondritis, Scheuermann's syndrome, polyarthritis, juvenile idiopathic arthritis[b] (onset at 3 years of age)	13	[a]225	c.334G>C; p.(Gly112Arg)	Exon 5
20	66	F	No	No	Lumbar spondylolisthesis, vitamin D deficiency, cavus feet, dorsalgia, rachialgia, L5 disc arthrosis	28	[a]175	c.1328C>T; p.(Ala443Val)	Exon 12
21	45	F	No	No	Paraesthesia in face and upper limbs; pain in elbows, hips and lower limbs; fatigue; muscle weakness; lower back cramps	10	[a]462	c.1135C>A; p.(His379Asn)	Exon 10

[a]PLP levels higher than the reference values. (–) denotes a negative result. Does not apply when PLP levels are in the range of the reference values. M, male; F, female. [b]disorder clasified as autoimmune disease.

Table 3: Clinical characteristics, ALP levels, PLP levels and genetic testing results in the pediatric population.

Patient	Age	Sex	Bone fractures	Dental abnormalities	Main diagnosis	ALP (a: 132–312 U/L; b: 75–312 U/L)	PLP (3.6–18 ng/mL)	Genetic testing results	Location
1	6[a]	M	No	No	Duchenne disease	104	[c]29	(–)	–
2	15[b]	M	No	No	Valgus feet	73	[c]118	c.17T>C; p.(Leu6Ser)	Exon 2
3	15[b]	M	No	No	Crohn's disease[d]	45	[c]45.5	c.498_500delCAC; p.(Thr167del)	Exon 6

[a]ALP reference range in males aged 2–9 years. [b]ALP reference range in males aged 15–16 years. [c]PLP levels higher than the reference values; (–) denotes a negative result. M, male. [d]Indicates autoimmune disease.

proposed diagnostic algorithm, the new estimated prevalence of mild HPP was almost double that previously estimated by our group (1/1,692 vs. 1/3,100).

Regarding ALP and PLP levels related to clinical manifestations in the adult population, we did not observe a link between the severity of clinical manifestations and lower levels of ALP. However, PLP levels could act as a marker of HPP severity, since patients with levels close to 200 ng/μL or higher showed more severe symptomatology (Patients 19, 21, Table 2).

The findings of our study indicate the great importance of establishing a good diagnostic algorithm for the diagnosis of HPP, considering appropriate reference values standardized by age and sex in the laboratory, together with the need to detect and report patients with persistently decreased ALP values in different analytical tests. Indeed, we propose a new algorithm capable of detecting most of the undiagnosed cases, which could improve the quality of life of up to 27,177 people only in Spain. Most importantly, our algorithm could avoid the negative effects of inappropriate treatments such as bisphosphonates, which lead to a worsening of HPP symptomatology. It is important to emphasize the need to perform genetic studies in patients with biochemical analytical values compatible with HPP even in the absence of clinical symptoms, since these patients could have asymptomatic HPP at the current stage, to avoid the use of these mentioned counterproductive therapies that worsen the patients' prognoses.

Moreover, it should be noted that despite no mutation in the *ALPL* gene being detected in four patients (patients 2, 3 and 9 (Table 2) and 1 (Table 3)), we cannot rule out an HPP diagnosis since their biochemical levels of ALP and PLP and main clinical manifestations were consistent with a potential HPP diagnosis. The cases of these patients could be explained by the presence of unidentified mutations in the noncoding region of the *ALPL* gene, since in clinical practice, genetic testing through Sanger sequencing sequences only the coding and flanking regions of the *ALPL* gene, so the promoter regions or intronic regions of the gene are currently not considered [37]. In this context, mutations in the noncoding regions can also lead to decreased protein activity due to alterations in alternative splicing processes, as we have observed in some patients in whom mutations in the intronic flanking regions have been identified (patients 13 and 14 (Table 2)). On the other hand, the existence of genetic variants in other factors involved in TNSALP regulation, such as RUNX2 and ENPP1 [31, 32], could affect ALP levels in these patients, leading to HPP development through alternative pathways. In this line, there are some diseases, such as acrodermatitis enteropathica, that lead to hypophosphatemia due to Zn^{2+} deficiency caused by a mutation in the SLC39A4 gene encoding the zinc transporter Zip4 [38]. Adequate Zn^{2+} levels are essential for TNSALP activity, so diseases other than HPP can directly affect TNSALP activity, leading to clinical manifestations related to HPP. In this context, we identified two subjects with hemochromatosis in our cohort. Hemochromatosis is a disease recently identified as a secondary cause of HPP due to the iron and ferritin inhibition of ALP activity [34, 36], but it is not currently included in the exclusion criteria considered in clinical practice, which should be updated. In this regard, on the basis of these findings, we have updated our HPP diagnostic protocol considering these secondary causes (hemochromatosis and acrodermatitis enteropathica) as exclusion criteria for HPP diagnosis.

Based on these considerations, HPP prevalence could be even higher than what we are detecting with the applied algorithm. In fact, our group is currently assessing these factors to deepen the understanding of the molecular pathways involved in HPP. In this context, because mutations in *ALPL* noncoding regions or in other TNSALP regulatory genes have been found, it would be advisable to improve clinical diagnostic protocols by including all known secondary causes of HPP and to perform complete sequencing of the *ALPL* gene through next-generation sequencing (NGS) to identify mutations in the noncoding regions or in the regulatory sequence of the gene in clinical practice [10, 39].

On the other hand, one of the most important findings of this study is the remarkable incidence of autoimmune diseases observed in patients with low decreased ALP activity. In this context, we found that 9/24 (37.5 %) participants (with persistent low levels of ALP based on our diagnostic algorithm) were affected by some type of autoimmune condition. For example, we found subjects affected by rheumatoid arthritis (patient 5, Table 2); dermatomyositis (patient 9, Table 2); psoriatic spondyloarthrosis and thrombocytopenic purpura (patient 10, Table 2); psoriasis (patient 12, Table 2); lupus (patient 13, Table 2); polyarthritis and synovitis, acne, pustulosis, hyperostosis, osteitis (SAPHO) syndrome (patient 14, Table 2), autoimmune thyroiditis (patient 15, Table 2); juvenile idiopathic arthritis (patient 19, Table 2); and Crohn's disease (patient 3, Table 3). Among them, considering only the subjects with some mutation in the *ALPL* gene identified by sequencing in our study, we observed the same incidence (37.5 %), since 6/16 subjects were affected with one of these autoimmune disorders (patients 10, 13, 14, 15, 19 – Table 2, and patient 3, Table 3). Since the prevalence of autoimmune disease in the general population worldwide is estimated to be between 3 and 8 % [40], the high proportion of HPP patients showing these comorbidities (four times higher than the general population) suggests that there might be a relationship between HPP and these conditions. This suggested link between low ALP levels and autoimmune involvement is an important finding not described in the scientific literature to date and has important clinical and therapeutic implications. According to these findings, our research group is currently investigating this issue to better characterize HPP disease and improve the management of the disease at the clinical level.

Regarding the limitations of the study, the main limitation is that it was carried out at a provincial level. Our prevalence calculations are based on an estimation through the extrapolation of national data, considering that the prevalence identified in Granada is similar to the prevalence in the other provinces of Spain. Therefore, to verify this estimated prevalence, a multicentric study of HPP in collaboration with other centers and clinical units from other cities is necessary. It would be interesting even at the European level to collaborate with different hospitals from Europe to establish a real European prevalence estimation. Another limitation of our study is that our algorithm excludes those patients with PLP levels in the range of the reference interval. This is a limitation because some cases of HPP-affected patients with PLP levels in the reference range have been recently found (results to be published). This implies that our algorithm should be updated considering finding. On the other hand, the main strength of our study is the design of an algorithm that has made it possible to detect a high percentage of cases that are usually undiagnosed through routine clinical practice (up to 80 %) in a simple and affordable way for any hospital or health care center. Thus, we can say that our proposed diagnostic algorithm shows high efficacy in detecting HPP and could be easily implemented in clinical practice.

In summary, the findings of this study have a great impact on clinical practice since the application of a diagnostic algorithm as effective as the one presented here can contribute to the correct diagnosis of a high proportion of patients with HPP who are currently undiagnosed or misdiagnosed. This is important since despite being a rare disease, when recalculating the HPP prevalence based on our results, the prevalence is twice as high as the last European estimated prevalence. At the therapeutic level, this knowledge may avoid the use of inappropriate treatments that may worsen the diagnosis of patients. On the other hand, it is worthwhile to deepen our understanding of the relationship found between HPP and autoimmune diseases to manage these diseases correctly.

Highlights

- Mild HPP may be twice as prevalent as previously estimated in Europe.
- Our proposed diagnostic algorithm performed well in detecting mild HPP cases, as 83 % of subjects genetically studied were found to suffer from HPP.
- Due to the high incidence of autoimmune diseases observed in HPP patients (~40 %), it could be interesting to deepen our understanding of the relationship of low ALP activity with autoimmune alterations.

Research ethics: This research complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013), and has been approved by Granada provincial Research Ethics Committee (CEI Granada) (ID Project: 0777-M1-20), on 8 May 2019.

Informed consent: Informed consent was obtained from all individuals included in this study.

Author contributions: M.M.-T., C.G.-F. and B.G.-F. designed the Study. B.G.-F., C.G.-F. and M.M.-T. directed the study's implementation. M.M.-T., V.C.-B., and J.M.G.-V. recruited the participant subjects. J.M.V.-S., V.C.-B., M.C.A.-L. and T.G.-C. carried out the study. B.G.-F., C.G.-F., J.M.G.-V., and F.A.-V. helped to prepare the methods section and interpret the findings., T.G.-C., S.G.-S., M.F.-M., L.M.-H. and J.M.V.-S. conducted the literature review and prepared the manuscript draft. B.G.-F., C.G.-F., M.F.-M. and M.M.-T.

reviewed and edited the final text. All authors have read and agreed to the published version of the manuscript. All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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Data availability: The data underlying this article cannot be shared publicly due to the privacy of individuals that participated in the study. The data will be shared on reasonable request to the corresponding author.

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