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Original Article

# Clinical, biochemical and genetic spectrum of low alkaline phosphatase levels in adults

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ABSTRACT

*Background:* Low serum levels of alkaline phosphatase (ALP) are a hallmark of hypophosphatasia. However, the clinical significance and the underlying genetics of low ALP in unselected populations are unclear.
*Methods:* In order to clarify this issue, we performed a clinical, biochemical and genetic study of 42 individuals (age range 20–77 yr) with unexplained low ALP levels.
*Results:* Nine had mild hyperphosphatemia and three had mild hypercalcemia. ALP levels were inversely correlated with serum calcium ($r = -0.38$, $p = 0.012$), pyridoxal phosphate (PLP; $r = -0.51$, $p = 0.001$) and urine phosphoethanolamine (PEA; $r = -0.49$, $p = 0.001$). Although many subjects experienced minor complaints, such as mild musculoskeletal pain, none had major health problems. Mutations in *ALPL* were found in 21 subjects (50%), including six novel mutations. All but one, were heterozygous mutations. Missense mutations were the most common (present in 18 subjects; 86%) and the majority were predicted to have a damaging effect on protein activity. The presence of a mutated allele was associated with tooth loss (48% versus 12%; $p = 0.04$), slightly lower levels of serum ALP ($p = 0.002$), higher levels of PLP ($p < 0.0001$) and PEA ($p < 0.0001$), as well as mildly increased serum phosphate ($p = 0.03$). Ten individuals (24%) had PLP levels above the reference range; all carried a mutated allele.
*Conclusion:* One-half of adult individuals with unexplained low serum ALP carried an *ALPL* mutation. Although the associated clinical manifestations are usually mild, in approximately 50% of the cases, enzyme activity is low enough to cause substrate accumulation and may predispose to defects in calcified tissues.


## 1. Introduction

The clinical significance of low serum levels of alkaline phosphatase (ALP) is unclear and consequently clinicians may not pay attention to them. In fact, low ALP may be an irrelevant finding or accompany various systemic disorders [1,2]. However, in some cases low ALP levels are the consequence of an underlying genetic disorder, hypophosphatasia, which influences tissue homeostasis, as well as drug responses.

Hypophosphatasia is a rare skeletal disorder due to a genetic defect in *ALPL*, the gene encoding the tissue-nonspecific (liver/bone/kidney) isoenzyme of ALP [3–5] or TNSALP. TNSALP is a homodimeric enzyme, with each monomer consisting of 524 amino acids. It connects to the cell membrane and functions as an ectophosphatase, hydrolyzing inorganic pyrophosphate (PPi), an inhibitor of mineralization, and other phosphate esters [6]. Apart from TNSALP, the ALP family comprises of three tissue specific ALPs: intestinal, encoded by *ALPI*; placental, encoded by *ALPP*; and placental-like 2 or germ cell, encoded by *ALPPL2* [7]. *ALPL* consists of 12 exons, of which the first and part of the second are noncoding. Several forms of hypophosphatasia have been described, mainly in children [6,8], with considerable variations in the disease spectrum, even within the same family [9].

Low serum ALP activity is frequently the first finding leading to a suspicion of hypophosphatasia. The diagnosis may be confirmed by genetic testing or by measuring other phosphorylated substrates, such as pyridoxal-5′-phosphate (PLP) or phosphoethanolamine (PEA) [8]. Hypophosphatasia in adults may either represent late manifestations of cases discovered in childhood, or adult onset forms [10–13]. Cases

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with less severe manifestations may pass unrecognized. However, they may have important consequences, including an increased risk of adverse effects after taking medication frequently used for treating osteoporosis, i.e. bisphosphonates [14]. On the other hand, the significance of lone low serum levels of ALP is unclear. Therefore, the aim of this study was to get a better understanding of the spectrum of low ALP levels in adults, by the active search of cases and the clinical, biochemical and genetic characterization of subjects with reduced serum ALP activity.

## 2. Materials and methods

### 2.1. Subjects

We reviewed serum ALP measurements in individuals aged 18 years and older during a 30-month period, in the Clinical Biochemistry laboratory, Hospital University Marqués Valdecilla, a tertiary facility serving a population of about 350,000 in Northern Spain. The clinical records of individuals with consistently low levels of ALP (see details in Results) were reviewed to exclude secondary causes of low ALP levels [2]. Thus, patients with renal failure, malnutrition or antiresorptive drugs, were excluded. Then, individuals with consistently low levels of ALP of unknown cause were contacted by telephone and offered to participate in the study. It included a clinical interview with a standard protocol, a physical examination and obtaining blood and urine samples for biochemical and genetic analyses. The study was approved by the Institutional Review Board and all participants gave informed written consent.

### 2.2. Biochemical and genetic analyses

ALP was measured by a colorimetric method in an Advia 2400 analyzer (Siemens Healthcare, Munich, Germany). The reference range in adults is 40–129 U/l. Serum calcium and phosphorus were also measured in an Advia 2400 analyzer (Arsenazo III and Phosphomolybdate methods, respectively),following manufacturer's instructions. The reference ranges were 8.1–10.4 mg/dl and 2.3–4.0 mg/dl, respectively. The bone isoenzyme of alkaline phosphatase (BAP) was measured by enzymoimmunoassay (Microvue BAP EIA kit, Quidel Corporation, San Diego, CA, USA); with a reference range of 12–43 U/l. PEA and PLP were measured at Reference laboratory (Barcelona, Spain). Urinary PEA was measured by HPLC (derivatization with o-phthalaldehyde and reverse phase high performance liquid chromatography with fluorescence detection); normal levels are below 70 μmol/g creatinine. PLP was determined by an enzymatic assay (VB6 enzymatic PoBühlmann, Bühlmann Laboratories AG, Switzerland); the reference range was 23–173 nmol/l.

The screening of the coding sequences and intron/exon boundaries of *ALPL* (NM_000478.4) was performed by direct sequencing. Primers were designed with the help of Primer 3 v0.4.0 software (http://bioinfo.ut.ee/primer3-0.4.0/) and SNPCheck V3 (https://secure.ngrl.org.uk/SNPCheck/snpcheck.htm) (Table 1). PCR products were sequenced using the BrightDye Terminator cycle kit (Nimagen, Nijmegen, The Netherlands) and run on an ABI3730XL Sequencer (Applied Biosystems, Foster City, CA). Conservation, in silico pathogenicity prediction and control population frequency analysis (ExoAC, Exome aggregation consortium) of the identified *ALPL* variants was carried out using Alamut V2.6–1 software (Interactive Biosoftware, Rouen, France) and MutPred (http://mutpred.mutdb.org/) and the evolutionary criteria proposed by Silvent et al. [7].We also consulted the *ALPL* mutation database (http://www.sesep.uvsq.fr/03_hypo_mutations.php) to check if any detected variant had been previously described, supporting its pathogenicity. We adopted the recommendations and guidelines of the American College of Medical Genetics and Genomics [15].

### 2.3. Statistical analysis

Comparisons between groups were analyzed by Mann–Whitney U tests or $Chi^2$ tests, as indicated. The correlation between biochemical parameters was estimated as the Spearman's correlation coefficient. All tests were 2-tailed and p-values less than 0.05 were considered as statistically significant.

## 3. Results

After searching the laboratory database (n = ~500,000 ALP analyses), we identified 12,546 serum ALP determinations in 8758 patients below the lower limit of the reference range (40 U/l). A more stringent 26 U/l threshold was chosen hereafter to increase specificity and avoid including individuals with occasional values below the standard normal range, likely to lack biological relevance. In 466 blood tests, performed in 181 patients, the enzyme level was <26 U/l. Among them, 130 individuals had persistently low levels (defined for the purpose of this study as at least one test result <26 U/l and none >40 U/l prior to the current study). After reviewing the clinical records, unexplained persistently low levels were found in 50 individuals. Of these, 42 unrelated subjects (10 men, 32 women) with an age range of 20–77 years (mean 50, median 49) were willing to participate in the study. A physician interviewed them and blood and urine samples were obtained for analysis.

Many subjects were asymptomatic or had mild ailments. A total of 24 complained of mild skeletal or muscular pain; 12 had suffered fractures (most were related to trauma and could not be necessarily considered due to bone fragility); two had a history of periarthritis and/or tendinopathy; nine had a diagnosis of osteoarthritis; nine, hypertension; one, coronary heart disease; and 13 had lost one or more teeth in the absence of trauma before 40 years of age.

Total ALP levels were positively correlated with BAP, and negatively with serum calcium ($r = -0.38$, $p = 0.01$), serum PLP ($r = -0.51$, $p = 0.001$), and urine PEA ($r = -0.49$, $p = 0.001$) (Table 2, Fig. 1). Serum PLP correlated with serum phosphorus ($r = 0.49$, $p = 0.001$) and urinary PEA ($r = 0.58$, $p < 0.001$), and negatively with BAP ($r = -0.54$, $p < 0.001$). Urinary PEA levels were within the reference range in all but one subject. Nine patients had mild hyperphosphatemia, whereas three had serum calcium levels slightly above the upper reference limit (Fig. 2).

**Table 1**
Primers sequences and amplicon sizes of the analyzed *ALPL* exons.

| *ALPL* exon | Forward (5´ > 3´) | Reverse (5´ > 3´) | Amplicon size (bp) |
|---|---|---|---|
| 2 | TCAGTTAACATCTGACCACTGC | CCCTCATCATACCCCATCTG | 215 |
| 3 | CACCTCCAAGTTCAGGCATT | AAACACCCTTCCTCCAGAGC | 352 |
| 4 | TACAGAGCCATGCCCAGTG | CTCTGGCTGCTGTCATGTTC | 326 |
| 5 | AGTCCCCATGGTGTGAGTGT | AAGCCTTTTCTAGCCCCTTC | 357 |
| 6 | AGGAGGCCTCTGGGACAC | GAGCCCATGGAGGAAAGATT | 364 |
| 7 | AAGTGTCCACACCATCTCCAG | GAGCCCATGGAGGAAAGATT | 382 |
| 8 | GATAGCTGCTGGGGTCAGTC | CTAAGTGGGCGTCAGGCTA | 247 |
| 9 | CCAGCCACCATACTCTACCC | ACCCCCAAACCAGTCAGTTC | 366 |
| 10 | TGGTGCTAGCTCAGAGTGGT | TGTCATTGAGTCCCCACCAT | 449 |
| 11 | AAGCCACCAAGGAGCCTAAT | GCTGACACCCTATTCCCAAG | 280 |
| 12 | CCTGGAAGGGAGATGGAAA | TGTGGGAAGTTGGCATCTGT | 399 |