



A Diagnostic Algorithm for Children with Low Alkaline Phosphatase Activities: Lessons Learned from Laboratory Screening for Hypophosphatasia

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Objectives To explore the role of laboratory screening for hypophosphatasia and propose a diagnostic pathway for children with low serum alkaline phosphatase (ALP) activities.

Study design A retrospective hospital-based study over an 8-year period was conducted to identify children younger than 16 years of age with low ALP activity (<100 U/L). Study-positive patients were contacted for repeat sampling, and those with persistently low ALP had plasma pyridoxal-5'-phosphate and urinary phosphoethanolamine measured.

Results Of 323 064 analyzed samples, 1526 had ALP activities <100 U/L. Most patients had transient hypophosphatasemia. Of 50 patients with last-recorded ALP <100 U/L, 32 were excluded given previous ALP >100 U/L. Eighteen were identified as study-positive. Of the 15 surviving children, 13 were traceable. Four had persistently low ALP activity on retesting, of whom 2 had raised pyridoxal-5'-phosphate and phosphoethanolamine concentrations and were subsequently tested for *ALPL* gene mutations; a 4-year-old asymptomatic girl with a novel homozygous *ALPL* missense mutation and a 23-year-old female with a heterozygous mutation. There was significant overlap in ALP activities between study-positive and 11 current patients with hypophosphatasia. We propose a diagnostic algorithm for children with low ALP activities based on clinical and biochemical variables.

Conclusions Patients with persistently low ALP activity require further clinical, biochemical, and radiological assessment for hypophosphatasia, even in the absence of clinical symptoms. The proposed diagnostic algorithm for children with low ALP will facilitate early detection of cases of hypophosphatasia, which, with the availability of enzyme replacement for hypophosphatasia, can be life-saving or avoid years of undiagnosed morbidity. (*J Pediatr* 2016;172:181-6).

Serum alkaline phosphatases (ALPs) are a group of glycoprotein enzymes that catalyze the hydrolysis of phosphoesters to release inorganic phosphate.¹ There are 4 different ALP isoenzymes, 3 tissue-specific ALPs (placenta, intestine, germ cell), and 1 tissue nonspecific ALP (TNSALP). The latter, encoded by the *ALPL* gene on chromosome 1, is widely expressed in various tissues including bone, liver, and kidney.² TNSALP cleaves extracellular substrates such as inorganic pyrophosphate (PPi) and pyridoxal-5'-phosphate (PLP).³

Reduced serum ALP activities are associated with a variety of conditions.⁴⁻⁷ Hypophosphatasia is a rare, inherited, potentially life-threatening bone disorder caused by inactivating mutations in the *ALPL* gene resulting in low ALP activity and accumulation of the enzyme substrates PPi, PLP, as well as an additional biochemical marker, urinary phosphoethanolamine (PEA).¹ PPi inhibits bone mineralization leading to skeletal abnormalities such as rickets, osteomalacia,⁸ fractures,⁹ and systemic complications.³ Based on age at presentation and severity of symptoms, hypophosphatasia is classified into perinatal lethal, prenatal benign, infantile, childhood, adult,¹⁰ and odontohypophosphatasia, which is limited to dental manifestations (no skeletal involvement).¹¹ Patients with the recessively inherited perinatal and infantile forms of hypophosphatasia come to medical attention before age 6 months because of pyridoxine-responsive seizures,^{12,13} failure to thrive, muscular hypotonia, hypercalcemia, nephrocalcinosis, craniosynostosis, fractures, or respiratory failure, and have a high mortality rate.¹⁴ However, the milder, usually dominantly inherited¹⁵ childhood and adult forms may have only subtle symptoms such as early tooth loss¹⁶ or chronic pain. The clinical phenotype varies, and all forms of hypophosphatasia can be associated with disability and/or poor quality of life.¹⁷

| | |
|--------|-------------------------|
| ALP | Alkaline phosphatase |
| PEA | Phosphoethanolamine |
| PLP | Pyridoxal-5'-phosphate |
| PPi | Inorganic pyrophosphate |
| TNSALP | Tissue nonspecific ALP |

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The diagnosis of hypophosphatasia is made on the basis of clinical, biochemical, and radiologic features.¹⁸ The hallmark biochemical feature of low serum ALP is typically associated with elevated serum phosphate, PLP, and urinary PEA levels, but diagnostic criteria for hypophosphatasia based on biochemical markers alone are not well established.³ In fact, asymptomatic carriers of recessive *ALPL* gene mutations also can have these biochemical abnormalities, although to a lesser extent.¹⁹ With the arrival of a novel enzyme replacement therapy for hypophosphatasia,²⁰ early detection of hypophosphatasia can be life-saving or avoid years of undiagnosed morbidity. Therefore, establishing better diagnostic criteria for children is vital for early detection and appropriate management of all forms of hypophosphatasia, including avoidance of inappropriate treatment.

We conducted a laboratory audit of children with low ALP activities in a large tertiary children's hospital. The aims were to explore potentially missed diagnoses in our institution, evaluate the role of laboratory screening for hypophosphatasia, and design a diagnostic algorithm for children presenting with either low ALP activity or clinical signs of hypophosphatasia.

Methods

We assessed all recorded serum ALP measurements between August 2004 and October 2012 at the Department of Clinical Chemistry, Birmingham Children's Hospital, United Kingdom. Appropriate pediatric ALP reference ranges are used in our hospital.²¹ Results from children under the age of 16 years were collated using the hospital's Telepath system. Patients with a low ALP concentration, arbitrarily defined as <100 U/L (based on levels from cases of severe hypophosphatasia in the literature),²⁰ in whom repeat ALP results either remained <100 U/L or where no repeat sample was obtained, were included. Patients >16 years of age were not included because ALP values <100 U/L fall within the normal reference range.²² As reference ranges for ALP activity are age dependent, patients were grouped into 5 age categories: neonates, 1 month-9 years, 10-11 years, 12-14 years, and 15-16 years.

Following identification of patients with persistently low ALP ("study positive"), approval from the hospital ethics advisory group was obtained to contact these individuals and arrange further investigations. All study-positive patients and their healthcare providers were contacted via letter and/or telephone requesting a repeat blood sample for ALP measurement. Urine PEA was performed in patients with persistently low ALP level on repeat sampling (below the reference range for age), and plasma PLP was measured in those patients with elevated urinary PEA concentrations. Those with low ALP and elevated PEA and PLP had radiographs and genetic testing of the *ALPL* gene performed.

Serum ALP activities were determined using a dye-based assay, which measures the enzyme activity by monitoring the rate of hydrolysis of p-nitrophenylphosphate to p-nitrophenol at 410/480 nm in the presence of magnesium on an

Olympus AU640 (Beckman Coulter, High Wycombe, United Kingdom). Urinary PEA was measured by amino acid quantification by ion exchange high performance liquid chromatography on a Biochrom 30+ amino acid analyzer (Biochrom Ltd, Cambridge, United Kingdom). Plasma PLP was measured by an external laboratory using high performance liquid chromatography following derivatization with fluorometric detection using a kit (Chromsystems, Munich, Germany).

To illustrate the overlap between ALP activities of study-positive patients and cases with hypophosphatasia, longitudinal ALP data from 11 children with confirmed hypophosphatasia, all patients currently managed at Birmingham Children's Hospital, were selected for comparison. Together with the outcome of this laboratory study, these comparative data contributed to the design of the proposed diagnostic algorithm.

Results

Over an 8-year period, 323 064 serum samples (from 62 285 patients) were analyzed for ALP activity, which showed a median concentration of 450 (range 17-29 600) U/L. Of the total samples, 1526 (0.47%) had ALP concentrations <100 U/L. The majority (75%) of these samples were from intensive care, 15% from ward inpatients, 5% from Accident and Emergency department, and remainder (5%) were from outpatient referrals. Sixteen samples from 4 known patients with hypophosphatasia, and 1317 samples (392 patients) with subsequent repeat ALP activity >100 U/L were excluded (**Figure 1**; available at www.jpeds.com).

Of the remaining 50 patients (193 samples), 17 (34%) had previously recorded normal ALP activity for age and 15 (30%) had previously recorded ALP activities of >100 U/L, thus, their low ALP activity on this occasion was considered secondary to acute severe illness. The remaining 18 (36%) study-positive patients never had ALP activities of >100 U/L. Three patients were less than one month old, 4 patients were 1 month-9 years of age, 2 patients were 12-14 years of age, and 9 patients were 15-16 years of age. A considerable overlap in serum ALP activities was observed between these 18 study-positive patients (median 84 [range 55-96] U/L) compared with the 4 patients with known hypophosphatasia (69.5 [41-94] U/L) (**Figure 2**). None of these 18 patients had had further diagnostic clinical or biochemical evaluations performed to exclude hypophosphatasia at the time of measurement.

On reviewing the medical notes of these 18 patients, 3 had died of severe head injury, fulminant sepsis, and acute renal failure. Thirteen of the 15 remaining patients were traceable and on repeat testing, 4 patients were found to have persistently low ALP activities for age with a median of 25.5 (range 24-65) U/L. Urine PEA was elevated in 2 (50%) of them, and on subsequent testing, their plasma PLP levels were also elevated. In view of the laboratory findings, genetic testing was performed that identified mutations in the *ALPL* gene in both patients.

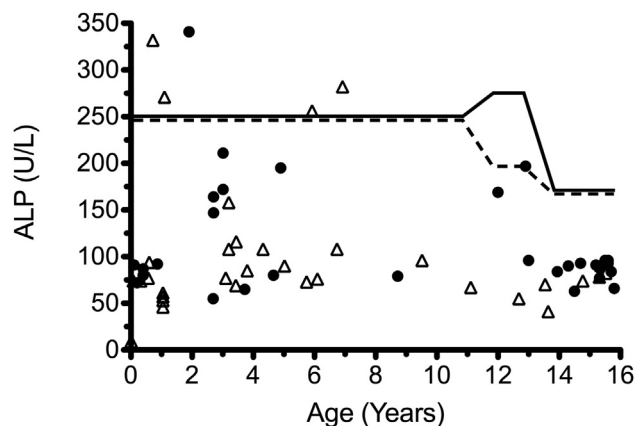


Figure 2. Scatter plot demonstrating the overlap in ALP activities in 11 current patients with confirmed hypophosphatasia at the study center (31 longitudinal samples, *triangles*) and the 18 study-positive patients (32 samples including retest values, *black circles*). The age- and sex-specific lower limit of ALP activity is depicted for males (*solid line*) and females (*dashed line*).

Characteristics of the New Cases of Identified Hypophosphatasia

These 2 patients, females aged 4 and 23 years at the time of re-testing, had a detailed history taken and were clinically examined. They had no history of fractures, no suspicious family history, no specific clinical features of hypophosphatasia, and knee radiographs were normal. The first patient (age 4 years), born to consanguineous (first-cousin) Pakistani parents, was completely asymptomatic, with normal current dentition. The only potential association with hypophosphatasia was a history of walking rather late (18 months), with an initial mild waddling gait that had quickly resolved. Genetic testing revealed a novel homozygous missense mutation c.715G>T, p.(Asp239Tyr) in exon 7 of the *ALPL* gene (Table). In silico analysis confirmed its pathogenicity, and both parents were heterozygous for this mutation. The second patient (now 23 years old) already had a known genetic diagnosis of minicore myopathy with rigid spine (compound heterozygous mutation in the *SEPN1* gene). She presented as a toddler with delayed walking and generalized muscle weakness. She developed scoliosis requiring corrective spine surgery. Our study found a heterozygous mutation in exon 6 of the *ALPL* gene, which she had inherited from her asymptomatic father (Table).

Proposed Diagnostic Algorithm for Patients with Low ALP Activities

Following the experience with this laboratory study, which had identified *ALPL* mutations in 2 of the 18 study-positive patients (11%), and based on the overlap in their ALP activities with an extended dataset of patients with current hypophosphatasia (Figure 2), the study team

Table. Laboratory results of the 4 study-positive patients with persistently low serum ALP activity on retesting, with further metabolic work-up for hypophosphatasia

| Age, y | Sex | Comorbidity | Initial ALP U/L (reference range) | Retest ALP U/L (reference range) | Serum phosphate (reference range 0.9-1.8 mmol/L) | Urinary PEA (normal <20 μmol/mol) | PLP (40-110 nmol/L) | Genetics |
|--------|-----|---|-----------------------------------|----------------------------------|--|-----------------------------------|---------------------|--|
| 4 | F | Nil | 92 (250-1000) | 211 (250-850) | 1.63 | 49 | 204 | Homozygous novel missense mutation in exon 7 of <i>ALPL</i> gene [c.715G>T, p.(Asp239Tyr)] |
| 23 | F | Minicore myopathy (compound heterozygous mutation in <i>SEPN1</i> gene) | 84 (200-730) | 24 (35-205) | 1.19 | 34 | 134 | Heterozygous mutation in exon 6 of <i>ALPL</i> gene [c.484G>A, p.(Gly162Ser)] |
| 18 | F | Solitary Kidney | 66 (170-460) | 24 (30-130) | 1.15 | 6 | - | - |
| 23 | F | Crohn's disease | 63 (170-460) | 27 (40-120) | 1.41 | 7 | - | - |

F, female.

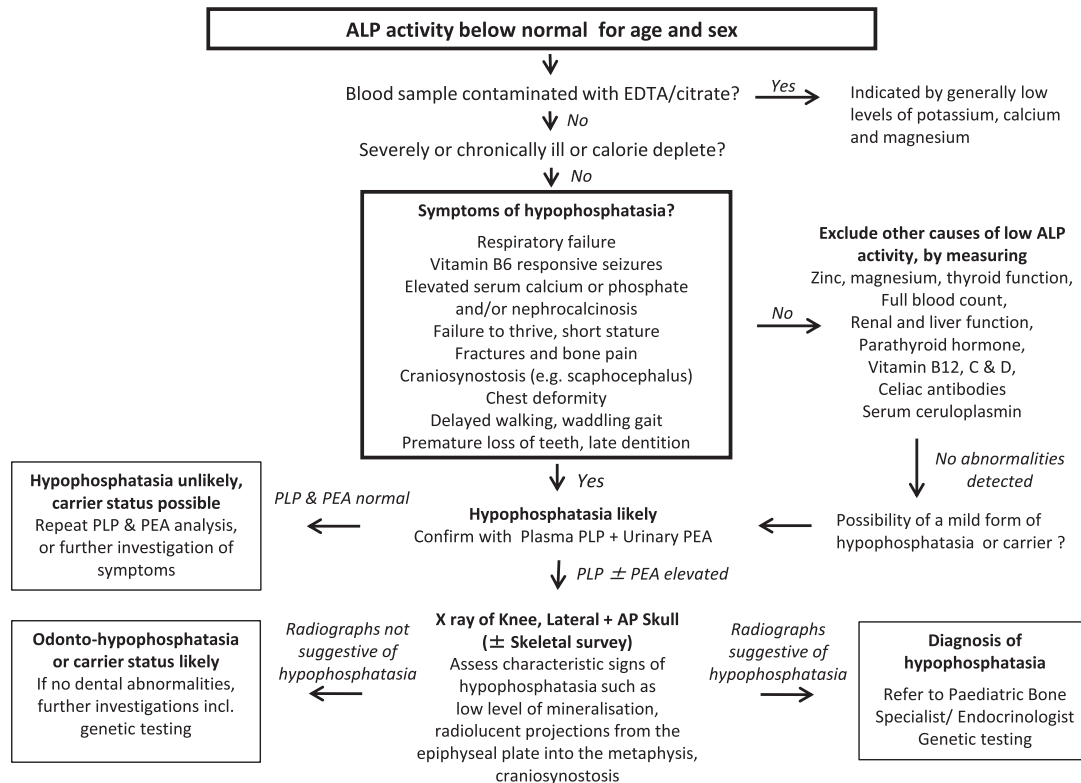


Figure 3. Diagnostic algorithm for the investigation of children presenting with low ALP activity and/or symptoms of hypophosphatasia. For patients with low ALP, a number of conditions such as nutritional deficiencies (protein/calorie, zinc, folic acid, magnesium, vitamin B6, B12, and vitamin C), vitamin D excess, hypothyroidism, hypoparathyroidism, celiac disease, recent significant blood transfusions, renal osteodystrophy, cardiac surgery and cardiopulmonary bypass, posthepatic resection and transplantation, achondroplasia, and Wilson disease, need to be excluded. AP, anteroposterior.

designed a diagnostic pathway for patients with low ALP concentrations with or without symptoms of hypophosphatasia (Figure 3). Use of the algorithm takes into account clinical symptoms, biochemical tests and radiologic signs, thus, facilitating diagnosis of hypophosphatasia or the other common differential diagnoses for children with low ALP activities, provided that appropriate pediatric reference data are used.

Discussion

The results of this study demonstrate that the vast majority of routine hospital patients with ALP activities <100 U/L have transient hypophosphatasemia, most likely because of low bone turnover caused by acute or chronic illness, or other factors and conditions outlined earlier.⁴⁻⁶ However, it was concerning that no child with persistently low ALP (“study positive”) had been further investigated for hypophosphatasia at the time of measurement, either clinically or biochemically by measuring TNSALP substrates (PLP or PPI) or urinary PEA. Therefore, the diagnosis of hypophosphatasia was missed in 2 of the 18 study-positive patients, neither of whom were clinically symptom-

atic for hypophosphatasia. Now that enzyme replacement for hypophosphatasia is becoming available,²⁰ early detection of hypophosphatasia is important to avoid misdiagnoses, long-term morbidity, complications, and death. This laboratory study also identified an asymptomatic child harboring a novel homozygous *ALPL* point mutation, which highlights our limited current understanding of genotype-phenotype correlations and the natural spectrum of hypophosphatasia.

We recognize that the overall proportion of samples with ALP activities <100 U/L was low (0.47%) and that the arbitrary cut-off used in the study could possibly have missed some milder forms of hypophosphatasia. The lower end of the reference range in children is age-, sex-, and assay-dependent (on our assay between 150 and 250 U/L [for those aged <16 years]). Therefore, it is likely that cases of milder hypophosphatasia and hypophosphatasia carriers are found just below, or even above (Figure 2), the lower end of the reference range. Our algorithm will be specifically useful for recognizing cases of milder hypophosphatasia, which are more common than severe cases.²³ In addition, a normal urinary PEA in isolation may not exclude hypophosphatasia, and, thus, we recommend combined PLP and PEA analysis.^{10,24}

This study also demonstrated a significant overlap in serum ALP activities between study-positive patients and those with known hypophosphatasia. Thus, a low ALP value by itself, in the absence of associated clinical symptoms is not diagnostic of hypophosphatasia and requires further investigations. Here, we propose a laboratory and clinical algorithm for patients with low ALP activities. In the first instance, appropriate sampling is essential because EDTA contamination of serum tubes are a source of error, leading to falsely low activity of ALP and other metabolites.²⁵ When low ALP activity is associated with biochemical features such as hyperphosphatemia and or hypercalcemia,¹⁸ and typical clinical signs, a diagnosis of hypophosphatasia can be made more confidently. In the absence of clinical signs of hypophosphatasia, other biochemical investigations need to be performed to exclude differential diagnoses. When low ALP activity is associated with elevated PLP and/or PEA concentrations, knee, lateral, and anteroposterior skull radiographs should be performed. Because of clinical heterogeneity,^{26,27} diagnosis of cases with milder hypophosphatasia, categorization into “classical” types based on age at diagnosis, and differentiation of hypophosphatasia from simple carriers status¹⁹ may be very challenging, as demonstrated by the patients discovered by our study. In such cases (ie, without clinical, radiologic, or dental signs), genetic testing will ultimately establish a diagnosis, but this facility may not be readily available across the globe.

Laboratories should establish procedures that prevent missed diagnoses, including the use of age- and sex-specific pediatric reference data and issue alerts to bring a low ALP activity to the immediate attention of the responsible clinician to trigger further diagnostic workup. Historically, clinicians are trained to recognize elevated ALP activities (for liver and bone disease). Therefore, education of clinicians and biochemists needs to be adapted to include diagnostic pathways for low ALP activity. The diagnostic work-up we propose is based on current knowledge of differential diagnosis of low ALP activity and recognized forms of hypophosphatasia. The phenotypic heterogeneity we describe, however, questions the current classification based on age at diagnosis. Increasing awareness among biochemists, pediatricians, and other health care professionals regarding hypophosphatasia in children is vital to facilitate not only early diagnosis and survival of severe cases of hypophosphatasia, but also for a better understanding of the natural course of the milder cases of hypophosphatasia. ■

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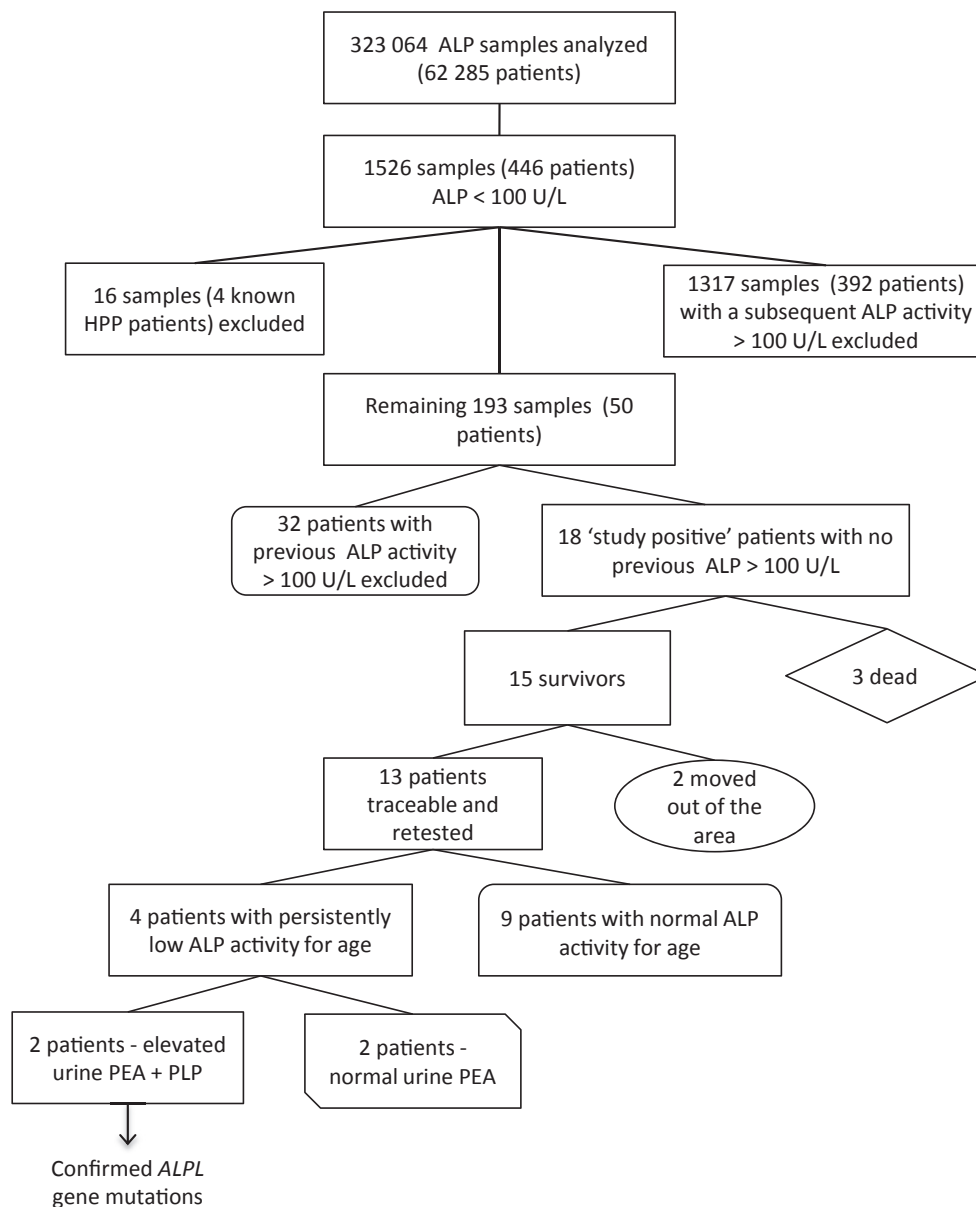


Figure 1. Flowchart showing study characteristics and patient selection.