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Issue: *Skeletal Biology and Medicine***Physiological role of alkaline phosphatase explored in hypophosphatasia**Michael P. Whyte<sup>1,2</sup><sup>1</sup>Center for Metabolic Bone Disease and Molecular Research, Shriners Hospital for Children, St. Louis, Missouri, USA.<sup>2</sup>Division of Bone and Mineral Diseases, Washington University School of Medicine at Barnes-Jewish Hospital, St. Louis, Missouri, USA

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**Hypophosphatasia (HPP) is the instructive rickets or osteomalacia caused by loss-of-function mutation(s) within TNSALP, the gene that encodes the “tissue nonspecific” isoenzyme of alkaline phosphatase (TNSALP). HPP reveals a critical role for this enzyme in skeletal mineralization. Increased extracellular levels of pyridoxal 5'-phosphate and inorganic pyrophosphate (PP<sub>i</sub>) demonstrate that TNSALP is a phosphomonoester phosphohydrolase and a pyrophosphatase that hydrolyzes much lower concentrations of natural substrates than the artificial substrates of laboratory assays. Clearly, TNSALP acts at physiological pH and “alkaline phosphatase” is a misnomer. Aberrations of vitamin B<sub>6</sub> metabolism in HPP revealed that TNSALP is an ectoenzyme. PP<sub>i</sub> excesses cause chondrocalcinosis and sometimes arthropathy. The skeletal disease is due to PP<sub>i</sub> inhibition of hydroxyapatite crystal growth extracellularly so that crystals form within matrix vesicles but fail to enlarge after these structures rupture. Trials of alkaline phosphatase replacement therapy for HPP suggest that TNSALP functions at the level of skeletal tissues.**

**Keywords:** ectoenzyme; inorganic pyrophosphate; osteomalacia; rickets; vitamin B<sub>6</sub>

**Introduction**

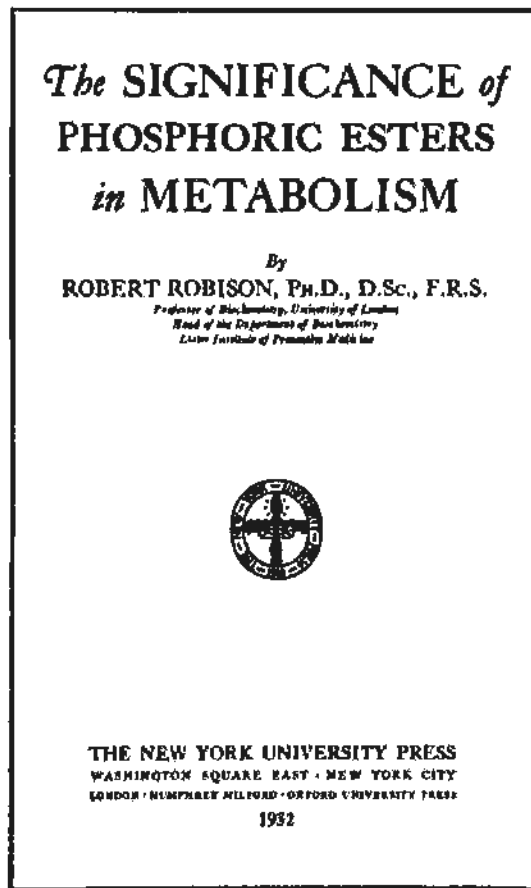
Alkaline phosphatase (ALP) was discovered in 1923 by Robert Robison who suggested this enzyme functioned in skeletal mineralization by liberating inorganic phosphate (P<sub>i</sub>) for hydroxyapatite (HA) crystal propagation (Fig. 1).<sup>1</sup> He found considerable phosphatase activity in the bone and cartilage of growing rats and hypothesized that mineralization followed hydrolysis of hexosephosphoric esters. In 1924, Robison and Soames demonstrated its peculiar alkaline pH optimum *in vitro*.<sup>1</sup> Robison recognized, however, that this was not physiological and never referred to “alkaline phosphatase.” He called his discovery “bone phosphatase.”<sup>1</sup>

Beginning in the 1930s, significant clinical insight came from measuring ALP in serum because hyperphosphatasemia usually indicates skeletal or hepatobiliary disease. Quantitation of serum ALP may still be the leading enzyme assay.<sup>2</sup>

Soon after Robison's report,<sup>1</sup> his hypothesis was challenged. ALP was abundant in noncalcifying tissues (e.g., liver, intestine, placenta), and extracellular fluid was supersaturated with P<sub>i</sub>.<sup>3</sup> Instead, other functions were proposed (see below).<sup>4–6</sup>

In the 1960s, electron microscopy rejuvenated Robison's hypothesis when the earliest HA crystals were discovered within “matrix vesicles” (MVs).<sup>7</sup> MVs seem to be buds of the plasma membrane of chondrocytes and osteoblasts and are rich in ALP. During “primary” skeletal mineralization, HA crystals appear and then grow within MVs. After MVs rupture, “secondary” mineralization proceeds as HA crystals enlarge and deposit into skeletal matrix.

By the 1990s, the postulated roles for ALP were many,<sup>4–6</sup> including hydrolysis of P<sub>i</sub> esters for the non-P<sub>i</sub> moiety, transferase action for synthesis of P<sub>i</sub> esters, regulation of P<sub>i</sub> metabolism, maintenance of steady-state levels of phosphoryl metabolites, and action as a phosphoprotein phosphatase.



**Figure 1.** Robert Robison's 1932 summary of his remarkable work concerning the importance of phosphoric esters in metabolism (New York University Press, New York).<sup>1</sup>

At plasma membranes, ALP perhaps conditioned transport of  $P_i$ , calcium, fat, protein, carbohydrates, and  $Na^+/K^+$ . Sequence analyses suggested coupling to other proteins, including collagen (see below). In the placenta, ALP bound the Fc receptor of IgG and perhaps transcytosed this immunoglobulin.

Additional roles also emerged for ALP in skeletal mineralization, including a plasma membrane transporter for  $P_i$ , an extracellular calcium ( $Ca^{2+}$ )-binding protein that stimulates calcium- $P_i$  precipitation and orients mineral deposition into osteoid, a  $Ca^{2+}/Mg^{2+}$ -ATPase, or a phosphoprotein phosphatase that conditions matrix for ossification.<sup>8</sup> Furthermore, certain domains seemed to bind ALP to types I, II, and X collagen in cartilage and bone. Nevertheless, an early theory gained preeminence; that

is, ALP hydrolyzes an inhibitor of calcification.<sup>3-6,9</sup> The principal candidate, inorganic pyrophosphate ( $PP_i$ ), impairs HA crystal growth and can be hydrolyzed by ALP.<sup>3,4</sup> In fact (see below), plasma and urine levels of  $PP_i$  are increased in hypophosphatasia (HPP).<sup>10</sup>

Now, Robison's hypothesis is proven; the "tissue nonspecific" isoenzyme of ALP (TNSALP) is essential for skeletal mineralization. Verification came from the discovery and subsequent characterization of the inborn error of metabolism, HPP.<sup>4-6</sup> In 1988, loss-of-function mutation(s) in *TNSALP* were first documented in HPP. The pathogenesis of the defective skeletal mineralization in HPP involves impaired hydrolysis of  $PP_i$ ,<sup>4-6</sup> representing the second regulator of mineralization anticipated by Robison.<sup>1</sup>

Ironically, nearly a century after the discovery of ALP, assay methods do not reflect Robison's appreciation of this enzyme.<sup>2,11</sup> In both clinical and research laboratories, ALP is measured using non-physiological alkalinity (e.g., pH 9.2–10.5) and high concentrations (millimolar) of artificial substrates (e.g., p-nitrophenylphosphate).<sup>2</sup> Furthermore, biologic specimens are diluted into buffers without  $P_i$ , although  $P_i$  competitively inhibits ALP.<sup>2,11</sup> These procedures ignore the pH optimum for ALPs being less alkaline for the lower concentrations of physiological substrates.<sup>2,6</sup>

To understand HPP and what it teaches us about ALP, a brief review of ALP genomic structure and protein chemistry is helpful.

### Genomic structure and protein chemistry of alkaline phosphatase

ALP (orthophosphoric-monoester phosphohydrolase, alkaline optimum, EC 3.1.3.1) is found ubiquitously in plants and animals.<sup>2</sup> In man, four ALP isoenzymes are encoded by four separate genes.<sup>5</sup> Three are expressed in essentially a tissue-specific distribution and form intestinal, placental, and germ cell (placental-like) ALP. The fourth is in all cells and designated tissue-nonspecific ALP (TNSALP).<sup>5</sup> Liver, bone, and kidney are especially rich in TNSALP, and the gene mapping symbol is *ALPL* (ALP-liver), although the function of TNSALP in bone, not in liver, is known (see below). The distinctive physicochemical properties (e.g., heat stability, electrophoretic mobility) of liver, bone, and kidney ALP are lost with glycosidase

exposure, reflecting “secondary” isoenzymes (isoforms) having an identical polypeptide sequence but different posttranslational modifications.<sup>5</sup>

The *TNSALP* gene is at the tip of the short arm of chromosome 1 (1p36.1–p34); the other ALP genes are found at the end of the long arm of chromosome 2 (2q34–q37).<sup>4</sup> *TNSALP* seems to be the ancestral gene.<sup>4</sup>

*TNSALP* exceeds 50 kb and contains 12 exons; 11 are translated to form the 507-amino acid residue *TNSALP*.<sup>12</sup> TATA and Sp1 sequences may be regulatory elements, but basal expression seems to reflect “housekeeping” promoter effects, whereas differential expression in tissues may use a posttranscriptional mechanism.<sup>5</sup> *TNSALP* has two promoters and two corresponding 5′ noncoding exons, 1a and 1b, resulting in two different mRNAs. Transcription from the upstream promoter (1a) is used in osteoblasts, whereas the downstream promoter (1b) is used in liver and kidney.<sup>4</sup> The tissue-specific ALP genes are smaller than *TNSALP*, primarily because of shorter introns. Amino acid sequences deduced from their cDNAs suggest 87% positional identity between placental and intestinal ALP but only 50–60% identity with *TNSALP*.<sup>5</sup>

The cDNA sequence of *TNSALP* reveals five potential N-linked glycosylation sites.<sup>12</sup> N-glycosylation is necessary for catalytic activity. O-glycosylation characterizes the bone, but not liver, isoform.<sup>5</sup>

In 2000, the crystal structure for placental ALP was detailed.<sup>13</sup> The deduced active site of *TNSALP* is encoded by six exons comprised of 15 amino acid residues and features a nucleotide sequence conserved in ALPs throughout nature.

The ALPs are Zn<sup>2+</sup>-metalloenzymes.<sup>2</sup> Catalysis requires a multimeric configuration of identical subunits with each monomer having one active site and two Zn<sup>2+</sup> atoms that stabilize the tertiary structure.

In tissues, ALPs are tethered to cell surfaces, probably as homotetramers.<sup>14</sup> In the circulation, ALPs are homodimeric.<sup>2</sup> *TNSALP* in dimeric form has αβ topology for each subunit with a 10-stranded β-sheet at its center.<sup>5</sup>

ALPs exhibit broad substrate specificities and pH optima *in vitro* depending on the type and concentration of phosphocompound undergoing catalysis.<sup>2</sup> PP<sub>i</sub> as well as phosphoesters can be hydrolyzed.<sup>15,17</sup> The reaction involves

phosphorylation-dephosphorylation of a serine residue. Dissociation of the covalently linked P<sub>i</sub> seems to be the rate-limiting step. In fact, P<sub>i</sub> is a potent competitive inhibitor of ALP.<sup>2,11</sup> However, P<sub>i</sub> may also stabilize the enzyme.<sup>16</sup> Catalytic activity requires Mg<sup>2+</sup> as a cofactor.<sup>2</sup>

Uncertainties persist about ALP biosynthesis. The human ALPs have a short signal sequence of 17–21 amino acid residues and a hydrophobic domain at the carboxyterminus.<sup>12</sup> They link to plasma membrane surfaces tethered to the polar head group of a phosphatidylinositol-glycan moiety<sup>18</sup> and can be released by phosphatidylinositol-specific phospholipase.<sup>14</sup> However, their precise interactions with phosphatidylinositol may differ. Intracellular degradation can involve proteasomes.<sup>19</sup>

Lipid-free ALP is found in the circulation. Yet the mechanism for its release from cell surfaces is unknown. The process could involve a C or D type phosphatidase, detergent action, proteolysis, membrane fractionation, or lipolysis.

In healthy adults, nearly all serum ALP consists of equal amounts of the bone and liver isoforms of *TNSALP*.<sup>4,5</sup> Infants and children, and particularly newborns and adolescents, have higher levels of the bone isoform.<sup>2</sup> Some people have intestinal ALP in the circulation after ingesting a fatty meal, but usually this is just a small amount of ALP (maximum 20%).<sup>2</sup> Placental ALP is typically expressed only during the last trimester of pregnancy.<sup>2</sup> Clearance of circulating ALP probably involves the liver.

## Hypophosphatasia

Subnormal extracellular concentrations of calcium and P<sub>i</sub>, or P<sub>i</sub> alone, cause nearly all types of rickets or osteomalacia.<sup>20</sup> HPP is an exception where the circulating levels are usually normal or elevated.<sup>4</sup> With the identification, beginning in 1988, of mutations within *TNSALP* causing HPP, Robison’s hypothesis was confirmed.<sup>1</sup> Additionally, *TNSALP* was shown to be necessary for development of primary teeth. However, undisturbed organs/tissues in HPP, particularly liver, led to the questioning of the significance for *TNSALP* elsewhere.<sup>4,6</sup>

## History

John C. Rathbun coined “hypophosphatasia” in 1948 when he reported an infant boy who died from

rickets and epilepsy and whose ALP in serum, bone, and other tissues was paradoxically subnormal.<sup>21</sup>

Understanding the physiological role of TNSALP was advanced by the discoveries of elevated levels of three phosphocompounds in patients. In 1955, excessive phosphoethanolamine (PEA) in urine provided a second biochemical marker for HPP. In 1965 and 1971, high levels of PP<sub>i</sub> in the urine and plasma,<sup>10</sup> respectively, revealed the mechanism for rickets or osteomalacia (see below).<sup>4,6,22</sup> In 1985, elevated plasma concentrations of pyridoxal 5'-phosphate (PLP), together with an understanding of vitamin B<sub>6</sub> (B<sub>6</sub>) metabolism, disclosed an coenzyme function for TNSALP and explained how the phosphocompounds accumulate extracellularly (see below).<sup>17</sup>

### Clinical features

HPP seems to affect all races.

Despite high levels of TNSALP in bone, cartilage, liver, kidney, and adrenal tissue (and at least some ubiquitously) in healthy individuals,<sup>2</sup> HPP appears to disrupt only "hard tissues" directly.<sup>4</sup>

Perhaps the most remarkable feature of HPP is its extraordinarily wide-ranging expressivity, spanning death *in utero* with nearly an unmineralized skeleton to difficulties with adult teeth without skeletal disease.<sup>4</sup>

Many (approximately 200) mutations in TNSALP, transmitted in various combinations by autosomal recessive inheritance or alone with dominant/negative effects over several generations by autosomal dominant inheritance, occur in HPP.<sup>23</sup> Furthermore, other genetic and nongenetic factors condition HPP expressivity, as illustrated by variable severity among siblings sharing TNSALP defects. Accordingly, the prevailing classification scheme for patients<sup>4</sup> remains a clinical one from 1957.

Five principal forms of HPP are usually discussed. The age at diagnosis because of skeletal disease distinguishes the perinatal, infantile, childhood, and adult types.<sup>4</sup> Individuals without skeletal findings but dental features only are said to have "odonto-HPP."<sup>4</sup> However, this nosology does not unambiguously classify all patients, and HPP is a wide-ranging continuum.<sup>4,6</sup>

The prognoses for these five major forms of HPP are determined by the skeletal complications. Typically, the earlier the signs and symptoms, the worse the outcome.<sup>4</sup>

### Perinatal hypophosphatasia

This most severe form of HPP is obvious in a neonate, manifests *in utero* with profound skeletal hypomineralization, and typically causes death at, or soon after, birth.

Skeletal radiographs readily distinguish perinatal HPP from the most severe forms of osteogenesis imperfecta or congenital dwarfism; the findings are diagnostic. In some stillborns, bones are nearly devoid of mineral; others have severe rachitic changes. In the skull, membranous bones may calcify only centrally, giving the illusion that cranial sutures are widely separated, although they may be functionally closed.

### Infantile hypophosphatasia

Skeletal disease manifests after birth but before 6 months of age<sup>4</sup> when there is failure to thrive. Then, rickets appears, leading to the diagnosis. B<sub>6</sub>-responsive epilepsy predicts a lethal outcome.<sup>24</sup> Rib fractures and chest deformity often lead to pneumonia. Acquired hypercalcemia is common and may explain recurrences of vomiting as well as nephrocalcinosis with renal compromise.<sup>4</sup>

The striking radiographic features are diagnostic. Cranial sutures may appear wide open but are the illusion from skull hypomineralization, and there can be "functional" craniosynostosis. Serial radiographs may reveal not only rickets but also gradual demineralization with fractures,<sup>24</sup> heralding a lethal outcome.<sup>25</sup> Alternatively, there can be spontaneous unexplained improvement.

### Childhood hypophosphatasia

This form has variable expressivity.<sup>4,26</sup> The diagnosis is made when bone disease is discovered after age 6 months. Premature loss of deciduous teeth (i.e., <5 years of age) is a major feature and results from hypoplasia of cementum.<sup>27</sup> Consequently, teeth slide out painlessly. Delayed walking and waddling reflect the degree of skeletal disease. Patients can complain of stiffness and pain and have appendicular muscle weakness consistent with a nonprogressive myopathy.

Radiographs usually show characteristic "tongues" of lucency that project from rachitic growth plates into irregular and widened metaphyses; these tongues are occasionally mistaken for infection or leukemia (Fig. 2). Premature fusion



**Figure 2.** Radiographic changes of childhood hypophosphatasia (HPP). (A) Metaphyseal irregularity, patchy osteosclerosis, and "tongues of radiolucency" characteristic of childhood HPP are present in the right distal radius and ulna of this boy at age 16 months. (B) Radiolucencies in childhood HPP that are concerning for leukemia are seen here in a different patient. Before referral, he underwent extensive oncologic evaluation at age 8 months because radiographs showed unusual "osteolytic" areas. Here, the metaphyses of the right distal radius and ulna are expanded and osteopenic but with ragged yet well-demarcated transition to irregularly hypomineralized diaphyses. The same process, although less striking, appears at his elbow. These abnormalities were subsequently explained by HPP.

of cranial sutures may raise intracranial pressure. Abnormalities may improve with growth plate closure after puberty (Fig. 3). Rarely, there is widespread bone marrow edema (Fig. 4).

#### Adult hypophosphatasia

Presentation is typically after middle age.<sup>4</sup> Not infrequently, however, patients manifest rickets and premature tooth loss at a young age and then become relatively healthy during their young adult life.

Osteomalacia manifests as recurrent, poorly-healing, metatarsal stress fractures.<sup>28</sup> With more advanced disease, aching and tenderness in the thighs is explained by femoral pseudofractures that will not heal unless they completely fracture. Early loss of secondary dentition is not uncommon, although

the cause is not understood. Calcium  $PP_i$  dihydrate (CPPD) deposition, occasionally with attacks of pseudogout, is from increased  $PP_i$  (see below). Some patients suffer degeneration of articular cartilage and "PP<sub>i</sub> arthropathy" and ossified ligaments resembling spinal hyperostosis.<sup>4</sup>

#### Odontohypophosphatasia

This form is diagnosed when there are dental manifestations but no evidence of rickets or osteomalacia.

#### Biochemical findings

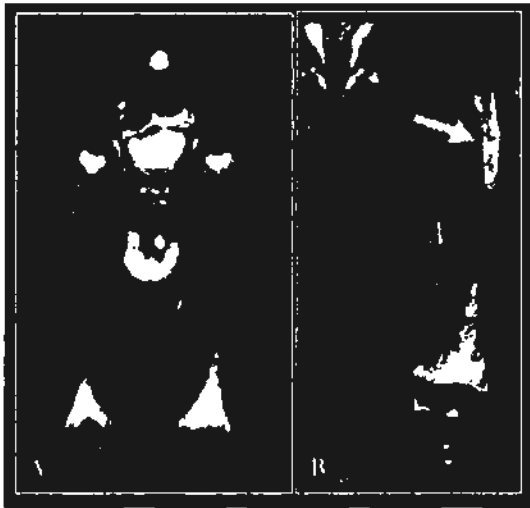
HPP can be diagnosed confidently from a consistent medical history and physical findings, subnormal serum ALP activity, and typical radiographic changes.<sup>4</sup>

Hypophosphatasemia can occur in other conditions.<sup>29</sup> Rarely, severe osteogenesis imperfecta and cleidocranial dysplasia manifest hypophosphatasemia due to little skeletal mass with impaired cellular processing of bone ALP or osteoblast hypofunction, respectively. In general, HPP severity correlates inversely with age-appropriate serum ALP activity.

In infantile HPP, hypercalcemia and hyperphosphatemia are common.<sup>24,30</sup> This seems largely from impaired calcium and  $P_i$  uptake by a poorly growing and mineralizing skeleton. Low serum parathyroid



**Figure 3.** Spontaneous improvement in childhood HPP at maturity. (A) The left knee of a patient with childhood HPP shows considerable "popcorn calcification" with metaphyseal sclerosis as well as irregularity of his growth plates at age 17 years. (B) The abnormalities improved spontaneously by age 20 years.



**Figure 4.** Bone marrow edema in infantile HPP. In two survivors of infantile HPP, marked bone marrow edema (BME) affects the skeleton. (A) T2-weighted, fat-suppressed magnetic resonance imaging of this 3-year-old boy shows BME in especially the proximal and distal femurs. There is also evidence of BME in the ischium. Distal femoral physeal distortion is consistent with severe HPP. (B) This 13-year-old girl has marked BME in the left femur where a Looser's zone is noted proximally (arrow).

hormone (PTH) levels follow. In childhood HPP, patients can have hypercalciuria. Serum 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, and PTH are typically normal unless affected by hypercalcemia or renal compromise.<sup>26</sup>

In childhood and adult HPP, serum  $P_i$  levels are above average, and approximately 50% of patients are distinctly hyperphosphatemic.<sup>4</sup> Enhanced renal reclamation of  $P_i$  underlies this incompletely understood finding. In some instances, suppressed PTH seems contributory.

Elevated urine levels of PEA support a diagnosis of HPP but are not pathognomonic.

Elevated plasma PLP is a sensitive marker for HPP.<sup>17</sup> Testing at commercial laboratories is sometimes ordered as "vitamin B<sub>6</sub>." Even odonto-HPP manifests high levels.<sup>17</sup> HPP severity correlates with plasma PLP,<sup>4</sup> although values overlap between clinical forms.

Assay of  $PP_i$  in plasma or urine is not commercially available. Urine  $PP_i$  levels are increased in most

HPP patients but can be unremarkable in mildly affected subjects.<sup>9</sup>

### Histopathological findings

Histopathological disturbances in HPP seem to involve only hard tissues directly.

Except in odonto-HPP, bone reveals defective mineralization<sup>26</sup> where ALP activity inversely reflects the degree of osteoid accumulation.<sup>26</sup> Features of secondary hyperparathyroidism are typically absent.<sup>26,31</sup> In growth plates and bone, the cellular sources of TNSALP (chondrocytes and osteoblasts) are present<sup>31</sup> but TNSALP activity is deficient.<sup>26</sup>

Electron microscopy of perinatal HPP has revealed a normal distribution of MVs containing HA crystals, although they lack ALP.<sup>26</sup> HA crystals fail to enlarge after MVs rupture.<sup>31</sup> Accordingly, secondary, but not primary, mineralization is compromised.

**Dentition.** In HPP, a paucity of cementum despite cementoblasts explains the premature tooth loss.<sup>27</sup>

Dentinogenesis also seems impaired, as revealed by big pulp chambers. The excessive predentin width, increased interglobular dentin, and impaired cementum calcification parallel osteoidosis in bone. Enamel is not impacted directly.

### Biochemical and genetic defects

#### TNSALP deficiency

Postmortem studies of lethal HPP revealed the TNSALP deficiency that defines this inborn error of metabolism. Profoundly low ALP levels are in liver, bone, and kidney but not in intestine or placenta.

#### Inheritance

Both autosomal dominant and autosomal recessive inheritance account for HPP. Perinatal and infantile HPP represent autosomal recessive disease; milder HPP results from autosomal recessive or autosomal dominant transmission of *TNSALP* defects.<sup>6,28</sup> Certain mutations have dominant-negative effects.<sup>6</sup>

#### TNSALP gene defects

In 1988, proof that HPP involves the candidate *TNSALP* gene came with discovery of a homozygous *TNSALP* missense defect in perinatal HPP. Now, all forms of HPP involve *TNSALP*, and all patients harbor one or two defective alleles.<sup>6</sup> A web site records the *TNSALP* mutations identified

in HPP patients worldwide (<http://www.sesep.uvsq.fr/Database.html>). Currently, approximately 200 *TNSALP* mutations are listed of which approximately 80% are missense.<sup>32</sup>

#### TNSALP structural defects

Many *TNSALP* mutations causing HPP change an amino acid conserved in mammalian TNSALPs,<sup>23</sup> including several in bacteria. Some disturb the catalytic pocket or structurally important metal ligand binding sites; others probably compromise dimer formation.<sup>4,23</sup> Some impair intracellular movement of TNSALP.<sup>23</sup>

#### Prognosis

Perinatal HPP is almost always fatal. Infantile HPP often features clinical and radiographic deterioration with approximately 50% of babies dying from respiratory compromise.<sup>24,25</sup> Sometimes, considerable spontaneous improvement occurs, especially if there is survival past infancy. Childhood HPP may improve when growth plates fuse. Unfortunately, recurrence of skeletal disease in adulthood is likely.<sup>28</sup> Adult HPP causes recurrent and lingering orthopedic difficulties.

#### Treatment

There is no established medical treatment, although several approaches have been attempted, including intravenous infusions of soluble ALP,<sup>33</sup> marrow cell transplantation,<sup>25</sup> and teriparatide administration.<sup>28</sup> Cortisone given to a few pediatric patients reportedly preceded normalization of serum ALP activity and radiographic improvement, but not consistently. Brief supplementation with Mg<sup>2+</sup> or Zn<sup>2+</sup> has been unsuccessful. Bisphosphonates (derivatives of PP<sub>i</sub>) could be ineffective or pose further problems.<sup>4</sup>

Following documentation that plasma PLP and urine PEA and PP<sub>i</sub> fell as placental ALP corrected the hypophosphatasemia of pregnant carriers of HPP,<sup>34</sup> i.v. infusions of purified placental ALP were used to correct hypophosphatasemia in a severely affected infant, but there was no clinical or radiographic improvement. This discouraging observation suggested even greater tissue requirements for ALP, or perhaps ALP must be within the skeleton and bound to plasma membranes for therapeutic efficacy.<sup>34</sup> Now, recombinant TNSALP is being evaluated. In 2008, TNSALP-null mice given

a bone-targeted human TNSALP from birth had no skeletal and dental disease or B<sub>6</sub>-responsive epilepsy.<sup>35</sup>

Because HPP patients are often hyperphosphatemic,<sup>4</sup> restriction and/or pharmacologic binding of dietary P<sub>i</sub> could theoretically be beneficial. P<sub>i</sub> competitively inhibits TNSALP<sup>2,11</sup> and suppresses TNSALP gene expression.

#### Physiological role of ALP explored in hypophosphatasia

Discovery in 1988 that *TNSALP* mutation caused HPP proved Robison correct.<sup>1</sup> The dental manifestations showed that TNSALP is also necessary for formation of primary teeth.

Electron microscopy of postmortem HPP bone and cartilage demonstrated that TNSALP enables skeletal mineralization. Secondary (extravesicular) not primary (vesicular) mineralization appears compromised.<sup>31</sup>

Defects in the cementum and dentin in HPP teeth seem analogous to those in bone. A prominent role for TNSALP during two critical phases of dental biomineralization—initiation and completion—has been proposed. In 2005, both cellular and acellular cementum formation were impaired but not mineralization of dentin.<sup>27</sup>

Although liver, kidneys, and adrenals are rich in TNSALP,<sup>2</sup> they seem to function normally in HPP (see below), except that renal reclamation of filtered P<sub>i</sub> is enhanced. This phenomenon does not seem to be always explained by suppressed circulating PTH levels.

It has been suggested that TNSALP deficiency might impair the biosynthesis of phospholipids causing pulmonary atelectasis in HPP; however, the respiratory problems are probably from rib cage deformities, perhaps accounting for hypoplastic lungs.

#### TNSALP substrates

Discoveries that PEA, PP<sub>i</sub>, and PLP accumulate endogenously in HPP were essential for elucidating the physiological role of TNSALP. Each phospho-compound was inferred to be a natural substrate. A preliminary study using <sup>31</sup>P magnetic resonance spectroscopy of HPP urine suggests at least several additional substrates.

### Phosphoethanolamine

In 1955, reports emerged that PEA is elevated in HPP urine and plasma. In 1968, essentially no renal threshold was demonstrated for PEA excretion.

Although its origin is uncertain, PEA is not considered a derivative of phosphatidylethanolamine, that is, not from plasma membrane breakdown. The major source of PEA is reported to be liver, which metabolizes PEA to ammonia, acetaldehyde, and  $P_1$ . Indeed, in one family with adult HPP, urine PEA correlated inversely with serum liver (but not bone) TNSALP. Now, PEA is understood to be part of the phosphatidylinositol-glycan linkage apparatus<sup>36</sup> and could be a degradation product from this tether for cell-surface proteins (see below).

### Pyridoxal 5'-phosphate

Discovery in 1985 that plasma PLP is elevated in HPP advanced our understanding of ALP.<sup>17</sup> The dietary forms of  $B_6$  are all converted to PLP in the liver. Organ ablation studies demonstrated liver as the principal source of plasma PLP, where >95% of PLP is coupled to albumin. Only a small amount circulates freely.

Like other phosphocompounds, PLP cannot cross plasma membranes but must be dephosphorylated to pyridoxal (PL). In cells, PL is rephosphorylated to PLP, which cofactors many enzymatic reactions. Ultimately, PLP is degraded primarily in the liver and excreted in urine.

In disorders that elevate serum ALP, plasma PLP is decreased.<sup>37</sup> Discovery of increased plasma PLP in HPP disclosed the reciprocal relationship between PLP and TNSALP and the control of  $B_6$  metabolism by ALP.<sup>17</sup> However, such effects may not be physiologically important because TNSALP controls extracellular, not intracellular, PLP. Elevated plasma PLP in HPP reflects a failure of extracellular hydrolysis of PLP.<sup>17</sup> Investigation of HPP suggested a generally inconsequential relationship because HPP patients do not have symptoms of  $B_6$  toxicity, such as peripheral neuropathy. Similarly, there are no signs or symptoms of deficiency, such as stomatitis, dermatitis, peripheral neuritis, anemia, or depression, in all but severely affected HPP babies with  $B_6$ -responsive seizures.<sup>17</sup> Urine levels of degraded  $B_6$  are unremarkable in childhood HPP,<sup>18</sup> where patients respond normally to L-tryptophan loading, a test for  $B_6$  deficiency (Whyte and Coburn, un-

published observation). In TNSALP-deficient HPP fibroblasts, the various forms of  $B_6$  are unremarkable. Finally, autopsy tissues from perinatal HPP contain normal levels of PLP and PL.<sup>18</sup> Accordingly, TNSALP seemed to function as an ectoenzyme,<sup>14,17</sup> as confirmed by studies of membrane attachment of porcine kidney ALP.<sup>36</sup>

Because TNSALP dephosphorylates PLP to PL extracellularly, plasma levels of PL could be low in HPP. However, only patients with extremely severe HPP have low plasma PL concentrations—other forms of HPP show normal or sometimes elevated levels.<sup>17</sup>

$B_6$  deficiency can cause renal lithiasis and epilepsy. Nephrocalcinosis in infantile HPP is likely due to hypercalciuria, but oxalate excess (a consequence of  $B_6$  deficiency) has not been searched for in HPP. Epilepsy in severe HPP has been associated with cranial deformity, intracranial hemorrhage, and periodic apnea; however, there may be another explanation. PEA caused seizures when given i.v. to a severely affected infant during a study of PEA metabolism.<sup>4,6</sup> In two patients with perinatal HPP, epilepsy, and plasma PL levels below assay sensitivity, pyridoxine did not correct seizures (personal observation).  $B_6$ -responsive seizures herald lethal HPP.<sup>24</sup> In fact, *tnsalp* knockout mice manifest epilepsy that requires pyridoxine administration to extend their lives (see below).<sup>38</sup>

Observations concerning  $B_6$  metabolism in HPP indicated an ectoenzyme role for TNSALP.<sup>17</sup> In 1980, discovery that porcine kidney ALP is bound to plasma membranes by phosphatidylinositol disclosed a mechanism for attachment of TNSALP.<sup>36</sup> In 1988, cultivated osteosarcoma cells, and then dermal fibroblasts from patients with infantile HPP, exposed to PLP and PEA confirmed this function (see below).<sup>14</sup>

### Inorganic pyrophosphate

Discovery in 1965 and 1971 that  $PP_1$  levels are increased in HPP urine and plasma<sup>10</sup> explained the defective skeletal mineralization.<sup>9</sup>  $PP_1$  was known to potently inhibit mineralization<sup>22</sup> by preventing the growth of HA crystals.<sup>9</sup>

Generation of  $PP_1$  extracellularly, presumably from ATP, is not hindered in HPP because nucleoside triphosphate  $PP_1$ -ase (NTP- $PP_1$ -ase, PC-1) activity is unremarkable in patient fibroblasts. Clearance of <sup>32</sup> $PP_1$  administered intravenously to adults



with HPP was markedly delayed (R.G.G. Russell, personal communication).

Consonant with *in vitro* effects of  $PP_i$ , minor excesses of  $PP_i$  may explain the precipitation of amorphous calcium phosphate and calcific peri-arthritis of some adults with HPP. Furthermore, ALP can dissolve CPPD *in vitro*.<sup>15</sup> This  $PP_i$ -ase activity seems unrelated to hydrolysis of phosphoesters. Thus, CPPD deposition leading to chondrocalcinosis, pseudogout, and  $PP_i$  arthropathy could be due to failed hydrolysis of  $PP_i$ .

### Circulating TNSALP

Several observations suggest that circulating (soluble) ALP is physiologically inactive. Infants with severe HPP who received plasma from patients with Paget bone disease or purified placental ALP demonstrated no significant clinical or radiographic improvement despite ALP sometimes increased to above normal levels. Such therapy failed to normalize urinary PEA or  $PP_i$  or plasma PLP.

Deficiency of TNSALP within the HPP skeleton seems to account for rickets and osteomalacia. In 1955, rachitic rat cartilage would calcify in HPP serum, yet HPP costochondral tissue would not mineralize in a synthetic calcifying medium or in serum from healthy children. Subsequently, transfection of ALP cDNA conferred both catalysis against  $P_i$  esters and mineralization in a calcification system.<sup>16</sup> There has been skepticism that such experiments reflect biomineralization because increased  $P_i$  in metastable solutions precipitates calcium  $P_i$ . Accordingly, it is probably important to augment ALP activity at the skeletal level to treat HPP. Marrow cell transplantation for infantile HPP preceded clinical and radiographic improvement despite essentially unaltered biochemical abnormalities, suggesting benefit from even small increments of ALP activity within the skeleton. Transient transfection studies of various TNSALP mutations also suggested that small differences in ALP activity could account for lethal versus nonlethal outcomes.<sup>23</sup>

### Hypophosphatasia fibroblast studies

Most of our insights concerning ALP came from investigations of patients with HPP. Before *tnsalp* knockout mice (see below), dermal fibroblasts in culture also proved useful.<sup>39</sup> Such cells can be profoundly deficient (<5% control) in ALP activity.

The ectophosphatase activity of TNSALP hydrolyzed extracellular PEA and PLP under physiological conditions.<sup>39</sup> Although some reports indicated that ALP conditions cell growth and differentiation perhaps by influencing the phosphorylation of nucleotide pools, HPP fibroblasts proliferate normally. TNSALP did not seem to be a phosphoprotein phosphatase acting at the plasma membrane.<sup>1,19</sup> Phospholipid composition and rates of  $^{32}P_i$  accumulation were normal.

### TNSALP knockout mice

Since 1995, *tnsalp* knockout mice have confirmed insight from HPP patients.<sup>4</sup> The *tnsalp* knockout mouse manifests the deranged  $B_6$  metabolism of HPP, causing lethal seizures from deficient  $\gamma$ -aminobutyric acid in the brain. With pyridoxine treatment, the epilepsy is controlled and the animals survive long enough to develop skeletal and dental disease. Two models recapitulate the infantile form of HPP remarkably well.<sup>38</sup> In 2000, the mineralization defect was reproduced with osteoblasts in culture. Defects in secondary skeletal mineralization were also confirmed by electron microscopy. Subsequently, double knockout mice showed that skeletal formation is essentially normal when both *tnsalp* and the  $PP_i$ -generating enzyme PC-1 (NTP- $PP_i$ -ase) are lacking, adding further support to experience with HPP patients.<sup>5</sup> Furthermore, *tnsalp* expression under control of the apo-e promoter in the liver of *tnsalp* knockout mice improved skeletal disease.<sup>40</sup> It is uncertain if *tnsalp* liberated in especially high amounts into the circulation and reaching bone itself explains the beneficial effects.

### Conflict of interest

The author declares no conflicts of interest.

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