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Review

Hypophosphatasia—pathophysiological understanding, preclinical data looking beyond the skeleton, and upcoming treatments

Jose Luis Millán*

Sanford Children's Health Research Center, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA 92037, United States
*Corresponding author: Jose Luis Millán, Sanford Children's Health Research Center, Sanford Burnham Prebys Medical Discovery Institute, 10901 North Torrey Pines Rd, La Jolla 92037, CA, United States (millan@sbpdiscovery.org).

Abstract

Hypophosphatasia (HPP) is a genetic disorder caused by loss-of-function mutations in the *ALPL* gene that encodes tissue-nonspecific alkaline phosphatase (TNAP), an enzyme essential for physiological skeletal/dental mineralization. In HPP, TNAP deficiency leads to the accumulation of extracellular pyrophosphate (PP_i), a potent inhibitor of calcification, resulting in skeletal and dental hypomineralization, with disease severity varying from the life-threatening perinatal and infantile forms to the milder later-onset forms that manifest in adulthood or only affect the dentition. Enzyme-replacement therapy based on recombinant mineral-targeted alkaline phosphatase (asfotase alfa) has been approved multinationally since 2015 for the treatment of pediatric-onset HPP, remarkably increasing the lifespan, their skeletal condition, and the quality of life of patients affected by the severe forms of HPP. However, non-skeletal symptoms remain an important clinical concern. As its moniker implies, TNAP is expressed in a large variety of tissues and cell types, and TNAP may be engaged in distinct metabolic pathways in each tissue. A better understanding of the cells expressing TNAP physiologically, the metabolic pathways involved, and the natural substrates of TNAP in each tissue will help design improved and/or alternative therapies to prevent/correct known or yet to be discovered non-skeletal manifestations of HPP.

Keywords: skeletal dysplasia, metabolic disease, enzyme-replacement therapy, gene therapy, small-molecule inhibitors, cell-based therapy, mouse models, pre-clinical testing, gene knockouts

Lay Summary

While the classic clinical signs of hypophosphatasia (HPP) are related to defective bone and tooth mineralization, non-skeletal symptoms represent a significant added burden for some patients. As novel therapies are conceived and implemented, it is crucial to keep in mind what aspects of HPP symptomatology we are not yet improving to better address those manifestations.

The disease

As expertly reviewed,[1] hypophosphatasia (HPP) features extraordinarily broad-ranging severity, but all patients carry 1 or 2 loss-of-function variants in their *ALPL* alleles. Hypophosphatasia is classified in the clinic according to whether there are dental manifestations alone (ie, without skeletal disease or other complications), and the age when any additional skeletal complications initially manifested. A recent article[2] summarized the features of HPP in the pediatric and adult patient populations, highlighting some overlapping features between the two age groups. The complications listed as specific for the perinatal-infantile-juvenile forms of HPP were growth failure, poor weight gain, rickets, premature loss of primary teeth (root intact), vitamin B_6-dependent seizures, flail chest, and osteochondral spurs. The complications listed as specific for the adult forms were osteomalacic fractures (metatarsal or femoral), osteoarthritis, loss of secondary teeth, chondrocalcinosis, and pseudogout. Importantly, however, there were a myriad of manifestations shared between the pediatric and adult groups. These shared manifestations included osteomalacia, bowing deformities, fractures, scoliosis, craniosynostosis, bone pain, muscle weakness, abnormal dentition, renal impairment, hearing loss, depression/anxiety, headaches, and fatigue. Clearly, HPP is an inborn error of metabolism that directly or indirectly affects multiple organ systems. We can explain many of those manifestations but not all, and as I will discuss below in this perspective article, we can prevent/ameliorate some of those manifestations with the current and developing treatments but not all. As we continue to press on with novel treatments, we need to also understand the long-term consequences of unresolved life-long deficits in other soft organs that may require tissue-nonspecific alkaline phosphatase (TNAP) for physiological functions and that the current and developing therapies are not addressing.

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The enzyme

Much of what we know about the structure of TNAP is based on homology studies done by modeling the TNAP amino acid sequence onto the crystallographic coordinates of the placental alkaline phosphatase (AP) isozyme structure.[3] Here, I will briefly describe the main structural features that can be affected by mutations and lead to the HPP disease. For an in-depth view of the structure of mammalian APs, I would refer the reader to my earlier treatise on the subject and references therein.[4] TNAP, as all mammalian APs, is an obligatory dimer, since the monomers are not stable and cannot mediate catalysis. The monomer-monomer interface displays a strong hydrophobic character and is crucial for the stability and enzymatic function of the dimer.[5] A flexible surface loop, "the crown domain," contains residues important for stabilizing the binding of uncompetitive TNAP inhibitors, a unique characteristic of APs and it also harbors a low-affinity collagen-binding motif.[6] The N-terminal alpha helix of each monomeric subunit reaches the active site of the contralateral subunit[7] and thus together with residues from the crown domain, help stabilize the dimeric structure and determine allosteric properties of the enzyme.[8] These structural and functional features explain why a single *ALPL* variant inherited from one parent can, in some cases, compromise the kinetic properties of the entire dimer (a dominant-negative variant) leading to TNAP insufficiency and generation-to-generation inheritance of HPP (dominant inheritance). Most other cases of HPP express two hypomorphic alleles for the enzyme dimer to be affected significantly (recessive inheritance and most often compound heterozygosity). Nevertheless, yet unknown modifier genes, epigenetic and non-genetic effects can significantly affect the expressivity and penetrance of the HPP phenotype. Three metal-binding sites (2 Zn^{2+} and 1 Mg^{2+} ions) surrounding the catalytic Ser residue are essential for TNAP enzymatic activity.[9] An additional metal-binding site, occupied by Ca^{2+}, has structural properties.[10]

To date, ~480 *ALPL* gene variants have been cataloged in the "*ALPL* Gene Variant Database" (https://alplmutationdatabase.jku.at/), providing a valuable foundation to attempt to understand genotype-phenotype associations. *ALPL* variants that alter residues at the monomer-monomer interface, the crown domain, the N-terminal arm, and the divalent cation-binding sites can all cause HPP.[11] Additionally, TNAP undergoes important post-translational modifications. TNAP is bound to the surface of plasma membranes and of matrix vesicles via a glycosylphosphatidylinositol (GPI) anchor that enables movement of the enzyme by enhancing membrane fluidity. This GPI anchor can be cleaved by the enzymatic action of phospholipases present in plasma membranes, partly explaining how TNAP is released into the blood and other biological fluids. Also, TNAP contains 5 N-linked glycosylation sites, at asparagine 140, 230, 271, 303, and 430, and some are required for catalytic activity.[12] The degree of glycosylation at these sites largely explains the different biophysical and kinetic properties of the TNAP "isoforms" produced by bone, liver, kidney, vascular cells, and others.[13]

Recently, the first crystallographic coordinates of TNAP were published.[14] The structure of the dimer confirmed features previously predicted and summarized above; however, the authors also described an octameric architecture for TNAP, generated by the tetramerization of dimeric TNAPs. It is hard to visualize how an octamer could be GPI-anchored on the membrane of cells and matrix vesicles. The authors suggest that such a multimeric arrangement could potentially stabilize TNAPs in the circulation. No evidence of the existence of such an octamer in the blood of healthy individuals was provided. The authors also mapped some *ALPL* variants associated with HPP to the dimer-dimer interphases of the octamer, but it is unclear how affecting multimer formation might contribute to the HPP disease. However, such a multimer could help explain some high molecular weight isoforms that have been described in blood associated with liver disease.[15] Two more TNAP structures have recently been determined. The group of David Magne (Lyon, France) confirmed the dimeric 3D structure for TNAP and mapped how phosphocholine (a newly uncovered substrate for TNAP) binds in an orientation opposite to that of pyrophosphate (PP_i) interacting with different amino acids in the active site of TNAP. The research group also used cryo-EM to demonstrate how binding of SBI-425,[16] a potent uncompetitive inhibitor of TNAP, effectively blocks access to the active site, preventing the dephosphorylation of both substrates.[17] The group of Lawrence Kazak (McGill University, Montreal, Canada) has determined the 3D structure of TNAP with phosphocreatine (PCr, another of its substrates) bound to the active site and confirmed the dimeric structure of the enzyme as well. This group has also identified for the first time a physiological allosteric activator of TNAP and mapped some HPP-causing *ALPL* variants to that allosteric site (personal communication—manuscript under revision).

Mouse models of HPP

The generation and characterization of mouse models of HPP proved indispensable for the probing of biochemical pathways involving TNAP function and the preclinical testing and optimization of established and potential novel treatments for HPP. I have reviewed some of those strains,[18] so I will now only briefly provide an update of the mouse models that are currently being used to test therapies (Table 1).

The (*Alpl*^{tm1Sor} and *Alpl*^{tm1Jlm}) alleles developed independently in the 1990s, have been the most used to examine the physiological consequences of a global TNAP ablation.[19] These *Alpl*$^{-/-}$ mice faithfully phenocopy infantile HPP, featuring failure to thrive, severely hypomineralized skeleton,[20] hypomineralized dentine[21] and enamel,[22] lack of acellular cementum,[23] and death prior to weaning, coinciding with severe seizures.[24,25]

The floxed *Alpl* allele that we generated enabled us to produce and characterize late-onset models of HPP, by conditionally inactivating TNAP under the control of the collagen 1 promoter (*Alpl*$^{Col1/Col1}$), affecting mineralization throughout the skeleton and teeth, or the *Prxx1* gene in the limb bud mesenchymal stem cells (*Alpl*$^{Prx1/Prx1}$), affecting primarily the appendicular skeleton.[26] It is this *Alpl*$^{Prx1/Prx1}$ model that we have consistently used to study the efficacy of adeno-viral administration of enzyme replacement[27,28] (see below) but we have also used an *Alpl*$^{-/Prx1}$ model where one *Alpl* allele is globally inactivated and the other conditionally inactivated. This model is somewhat more severe and facilitates evaluating skeletal improvement when we used ENPP1 inhibition to lower their high levels of PP_i (ASBMR 2025; FRI&SAT-282) or when we examined the consequences of superimposing CKD as a comorbidity onto HPP (ASBMR 2025; FRI&SAT 293—manuscript under review). DeMambro et al.,

Table 1. Murine models of HPP spanning the severity of the disease (modified from Millán and Whyte[18]).

Manifestations	*Alpl*$^{-/-}$	*Alpl*$^{Prx1/Prx1}$ or *Alpl*$^{-/Prx1}$	*Alpl*$^{Col1/Col1}$	*Alpl*$^{+/A116T}$	*Alpl*$^{Wnt1/Wnt1}$
Seizures	Yes	No	No	No	Np
Perinatal death	Yes	No	No	No	Mo
Rickets or osteomalacia	Yes	Yes	Yes	No	No
Joint defects	Yes	Yes	Yes	No	No
Alveolar bone defects	Yes	Yes	Yes	Yes	Yes
Acellular cementum deficits	Yes	Yes	Yes	No	Yes
Cellular cementum deficits	Yes	Yes	Yes	Yes	Yes
Dentin deficits	Yes	Yes (incisors)	Yes	Yes	Yes
Enamel defects	Yes	?	?	Yes	Yes (incisors)
Craniosynostosis	Yes	No	No	No	Yes
Nephrocalcinosis	Yes	No	No	No	No
Dyslipidemia	Yes	?	?	?	?
Muscle fiber alterations	Yes	?	?	?	?
Grip strength issues	Yes	Yes	?	?	?
Endurance issues	Yes	Yes	?	?	?
Metabolic rate issues	?	Yes	?	?	?
Thermogenesis	Yes	?	?	?	?

Abbreviation: HPP, hypophosphatasia.

(manuscript in preparation) have recently used this model (which they called PAKO mice) to evaluate endurance, grip strength, and metabolic changes in HPP (ASBMR 2024; SAT-LB 562) and evaluate the efficacy of Ilofotase alfa (ASBMR 2025; 1020).

Our early attempts to develop a mouse model of odonto-HPP, by introducing the A116T dominant negative *ALPL* variant, led to a disappointingly mild phenotype,[29] and we have not since used this model for any treatment studies. However, we recently published the generation of a neural crest-specific conditional TNAP deletion via breeding the *Alpl*$^{flox/flox}$ mice to a *Wnt1*^{Cre2} mouse to conditionally delete TNAP in ectomesenchymal cells that make dentine, cementum, periodontal ligament, and alveolar bone, but without causing any changes in the appendicular skeleton.[30] Defects were noted in the neural crest-derived intersphenoid synchondrosis of the cranial base and the mandibular condyle of *Alpl*$^{Wnt1/Wnt1}$ mice, and extraction of maxillary molars demonstrated profound alveolar bone healing defects that were partially rescued by enzyme replacement.[30] We will continue to use this newly developed model of craniofacial and dentoalveolar defects phenocopying odonto-HPP to test novel therapies. A caveat with the mouse models is that, unlike in humans, teeth are continuously erupting and thus do not entirely recapitulate human dentinogenesis. In this regard, a new sheep model of HPP, generated via CRISPR/Cas9-mediated knock-in of a missense mutation (c.1077C > G; p.I359M) closely recapitulates the dentoalveolar defects reported in individuals with HPP.[31]

Known substrates and the associated phenotypic changes in HPP

Some physiological substrates have been known to be pathologically elevated in HPP. Others have only recently been identified in pre-clinical studies. I will briefly summarize what is known about their physiological roles (Table 2).

Pyrophosphate (PP_i)(mineralization): Extracellular PP_i is a potent inhibitor of mineralization and is considered the body's natural water softener[32] as it efficiently prevents inappropriate soft tissue calcification. In blood, PP_i concentrations are in the μM range, while inorganic phosphate (P_i) levels are in the mM range and, as a result, small changes in the PP_i/P_i ratio have profound implications for the control of soft-tissue calcification. TNAP plays the crucial role of restricting the concentration of extracellular PP_i to maintain a PP_i/P_i ratio that is permissive for normal bone mineralization, and I have described this mechanism in detail in a previous review.[33] To paraphrase from that review, the failure of HPP bones to calcify results from two biochemical events, (1) a block in the propagation of hydroxyapatite in the extracellular matrix because of accumulated levels of PP_i from the lack of TNAP's pyrophosphatase function, and (2) inadequate local generation of P_i from ATP in the milieu surrounding the matrix vesicles. HPP patients and *Alpl*$^{-/-}$ mice retain the ability to initiate intravesicular mineral formation and contain hydroxyapatite crystals due to the presence of phosphoethanolamine (PEA)/phosphocholine phosphatase 1 (PHOSPHO1) generating P_i within matrix vesicles. *Phospho1*$^{-/-}$ mice display growth plate abnormalities, spontaneous fractures, bowed long bones, osteomalacia, and scoliosis in early life, consistent with an HPP-like phenotype.[34] In a very revealing genetic experiment, we found that the [*Phospho1*$^{-/-}$; *Alpl*$^{-/-}$] double knockout pups were embryonic lethal with a complete absence of skeletal mineralization despite the normophosphatemia/hyperphosphatemia manifested by the individual knockout strains, respectively. This strongly indicated that the availability of free circulating P_i is not sufficient to initiate mineralization and that TNAP must be involved in generating P_i in the vicinity of matrix vesicles. Indeed, a P_i-generating function had been proposed for TNAP since its discovery in bone by Robison in 1923,[35] and TNAP's ability to hydrolyze ATP to generate P_i is now well documented.[36]

ATP/ADP/AMP (systemic effects): The high ATPase activity of TNAP also shows that besides generating P_i for mineralization, it is also able to progressively generate ADP, AMP, and adenosine.[36] Cleavage of pro-inflammatory ATP and production of anti-inflammatory adenosine by high plasma TNAP activity in neonates contribute to enhancing an anti-inflammatory purine metabolism profile in newborn blood.[37] Furthermore, TNAP is 1 of 3 enzymes involved in purine metabolism, contributing anti-nociceptive adenosine for murine somatosensory dorsal root ganglia neurons and the dorsal spinal cord[38] and reduce extracellular ATP levels to enable axonal growth in hippocampal neurons.[39] Altered purinergic signaling in *Alpl*$^{-/-}$ mice resulting from an increased ATP/adenosine ratio caused by TNAP deficiency

Table 2. Natural substrates of TNAP, and their physiological and pathophysiological roles.

TNAP substrates	Biochemical pathway(s)	Systemic manifestations
PP_i	Generate a permissive PP_i/P_i ratio during physiological mineralization	Skeletal/dental hypomineralization
ATP, ADP, AMP	Generation of P_i; generation of adenosine	Skeletal/dental mineralization; Purinergic signaling regulation; Axonal growth; Nociception; Muscle weakness?
Pyridoxal 5' Phosphate (PLP)	Cofactor of ~ 160 enzymes; Inhibits P2rX7 activity	Seizures; spinal cord myelination; Mitochondria dysfunction? lipolysis?. Muscle weakness?
Phosphocreatine	Futile creatine cycle	Brown fat thermogenesis; cold adaptation?
Phosphocholine/Phosphoethanolamine	Dephosphorylation to choline and ethanolamine	Metabolism of triglycerides and cholesterol; liver steatosis
Bacterial endotoxins (LPS, others)	Detoxification via dephosphorylation	Blood-brain barrier integrity; sepsis, nephrocalcinosis?
Phosphorylated osteopontin	Inhibition of mineralization	Skeletal hypomineralization, immunoregulation?
Phospho-tau	Dephosphorylation of p-Tau	Reduced anxiety behavior; increased memory capacity and life expectancy during taupathies

Abbreviation: TNAP, tissue-nonspecific alkaline phosphatase.

may contribute to the seizures, hyperalgia, and allodynia seen in *Alpl*$^{-/-}$ mice, and may be part of the mechanism of pain experienced by HPP patients.

Pyridoxal-5'-Phosphate (PLP) (systemic effects): Another natural substrate of TNAP discovered by studying HPP patients is PLP, the major circulating form of vitamin B6.[1] Vitamin B6, mediated by its various vitamers (PLP, pyridoxal, pyridoxamine, and pyridoxine), is a co-factor in more than 160 biochemical intracellular reactions,[40] and more specifically a coenzyme in the catabolism of various aminoacids, and in the decarboxylations necessary for neurotransmitter generation, including dopamine, serotonin, histamine, taurine, and gamma-aminobutyric acid. As summarized earlier,[18] dephosphorylation of PLP to pyridoxal is one important function of TNAP as only the non-phosphorylated vitamers can enter cells, and once inside the cells, these non-phosphorylated vitamers are converted back to PLP to be used as a coenzyme for various enzymatic pathways. TNAP deficiency leads to increased plasma PLP levels but low PLP levels in the central nervous system in HPP patients, leading to decreased concentrations of serotonin and gamma-aminobutyric acid, which explains in part the severe seizures hours before or immediately preceding the death of *Alpl*$^{-/-}$ mice prior to weaning.[24,25] A very revealing double genetic experiment showed that ablating the purinergic receptor gene *P2rX7* in the *Alpl*$^{-/-}$ mice resulted in double knockout mice with extended lifespan that still succumbed to their HPP disease but without experiencing the seizures.[41] The authors found that PLP blunts the function of P2rX7, a receptor known to respond to high levels of ATP. The interpretation of those data suggests that the seizures in the *Alpl*$^{-/-}$ mice are due to 2 main biochemical changes: (1) an accumulation of ATP, resulting from deficient TNAP activity, that stimulates P2rX7 receptor activity, and (2) reduced blunting of P2rx7 activity due to the diminished concentrations of PLP in the CNS. This implies that drugs that target the activity of the P2rX7 receptor might potentially be used to help control seizures in severely affected HPP infants.

A comprehensive book by Fonta and Négyessy[42] (and references therein) recounts the large amount of evidence to date on the expression of TNAP in the developing murine neural tube and certain areas of the mature brain. In fact, *Alpl*$^{-/-}$ mice display a significant decrease in the white matter of the spinal cord with ultrastructural evidence of cellular degradation around the paranodal regions and a decreased ratio and diameter of the myelinated axons.[43,44] Some of these features are reproduced in wild-type animals fed a Vitamin B6 deficient diet, while conversely, they improve in *Alpl*$^{-/-}$ mice fed a diet rich in pyridoxal.[43] Could these myelination defects contribute to the mechanism(s) of pain in HPP?

Phosphocreatine (PCr) (thermogenesis - mitochondrial function): While TNAP is well known to be present on the cytoplasmic membrane of cells and on the outer surface of matrix vesicles during biomineralization, an unexpected finding described TNAP to be localized in the mitochondria of brown fat cells, where it acts as a potent PCr phosphatase to initiate a futile cycle of dephosphorylation and phosphorylation.[45] The paper describes that TNAP expression is powerfully induced when mice are exposed to cold conditions, and that inhibition of TNAP in isolated mitochondria using the pharmacological inhibitor SBI-425 leads to a loss of creatine cycling. The data indicated that, despite the absence of any identifiable mitochondrial targeting sequence, TNAP was tethered to mitochondrial membranes via a GPI-anchor and was localized to the inner mitochondrial membrane and in the intermembrane space. Furthermore, genetic ablation of TNAP in adipocytes reduced whole-body energy expenditure and led to rapid-onset obesity in mice, without changes in activity or feeding behavior. Pilot measurements in my laboratory examining 11- to 12-d-old *Alpl*$^{-/-}$ pups showed a decrease of ~1.4°C in body surface temperature compared to heterozygous and Wt siblings (unpublished results). Does this PCr-mediated mechanism, discovered in mice, suggest that cold adaptation issues might occur in HPP patients? There is no mention of changes in body temperature in HPP patients in the scientific literature, but anecdotal accounts from HPP patients might point to difficulties in cold adaptation. If proven to be part of the systemic presentation of HPP, does asfotase alfa treatment prevent or reverse such a symptom?

The data on the role of TNAP in brown adipose tissue prompted an investigation into the potential mechanism that might explain the lipodystrophy at the sites of injection that HPP patients sometimes experience with the almost daily injections of asfotase alfa. During her recent thesis defense, Vicky DeMambro (Cliff Rosen's laboratory—unpublished) showed that both the *Alpl*$^{-/-}$ and the *Alpl*$^{-/Prx1}$ models of HPP show reduced amounts of inguinal white adipose tissue accumulation and decreased mitochondrial function pre and post adipogenic differentiation, coupled with changes in mitochondrial dynamic genes. It is conceivable that local accumulation of asfotase alfa at the site of injection could cause the opposite

effect, driving an excessive lipogenic response. This hypothesis merits further investigation.

Another issue related to the possible impairment of mitochondrial function appears to be the muscle weakness experienced by HPP patients, and that resolves or improves with asfotase alfa treatment.[46] One earlier report described the presence of TNAP in the endoplasmic reticulum of the cardiac sarcomere in mice,[47] but to date there are no reports of TNAP being expressed in skeletal muscle. However, a paper has documented a significant reduction in the muscle length, mass, and cross-sectional area of the extensor digitorum longus muscle isolated from 14-d-old *Alpl*$^{-/-}$ mice.[48] In the same study, histological studies revealed an alteration in muscle fiber type composition with an increase in type II and a decrease in type I muscle fibers in the gastrocnemius muscle. *Alpl*$^{-/-}$ mice had also less nicotinamide adenine dinucleotide dehydrogenase positive fibers, pointing to a decrease in oxidative fibers.[48] Muscle strength and motor coordination tests were also carried out in 14-d-old *Alpl*$^{-/-}$ pups in another study, and the authors found a significant reduction in the ratio of grip strength/BW and also fell off an inverted screen earlier than those compared to Wt unaffected siblings.[49] Furthermore, data presented at ASBMR 2024 (SAT-LB 562) indicated that *Alpl*$^{-/Prx1}$ mice showed muscle weakness and decreased endurance coupled with a decrease in energy expenditure, when subjected to graded treadmill exercise tests on a metabolic treadmill, a standard wire hang, and a grip strength test. *Alpl*$^{-/Prx1}$ mice exhibited a 75% decrease in hangtime vs. controls and a 45% decrease in grip strength.

Could the muscle weakness improvement resulting from enzyme replacement reflect a direct effect of asfotase alfa homing into the mitochondria? It seems hard to visualize that such a large molecule as asfotase alfa (1452 amino acids for the dimer) would be able to enter the mitochondria, and furthermore the presence of a hydrophilic D_{10} motif would likely preclude this biologic from homing to the proper mitochondrial location. Most likely, the rapid improvement in muscle function would have to be due to the normalization of the concentration of one or several of the soluble substrates of TNAP. The likely candidates are either PP_i, ATP, or PLP. Toxicity by PP_i is a possibility that will need to be investigated. The interdependent role of ATP and P2rX7 should also be looked at. Given that a third of all PLP-dependent enzymes are localized in the mitochondria,[50] in my view, it is likely that the effects of TNAP deficiency (or excess) on adipogenesis and the culprit in the muscle weakness phenotype is the depletion of PLP in the mitochondria. Thus, asfotase alfa treatment, just as it resolves the PLP-associated perinatal seizures in the *Alpl*$^{-/-}$ mice and severely affected HPP patients, should be expected to correct the intracytoplasmic and intramitochondrial PLP deficiency, helping resolve the muscle phenotype.

Phosphoethanolamine (PEA) and phosphocholine: PEA has long been considered a substrate and a biochemical marker of HPP[1]. Looking back at the first published paper of my career, in collaboration with Drs Louis V. Avioli and Michael P. Whyte,[51] we had found that plasma PEA levels inversely correlated with blood levels of the liver but not the bone isoform of TNAP, suggesting altered hepatic metabolism as responsible for the increased urinary levels of this analyte. The group of David Magne recently investigated whether TNAP inhibition could prevent plaque calcification in atherosclerotic ApoE-deficient mice[52] and found that TNAP inhibition not only reduced vascular microcalcifications but also impacted the liver, reducing blood triglyceride and cholesterol levels, thus impairing the entire process of atherosclerotic plaque development.[52] Using an NMR metabolomics approach, the authors identified phosphocholine as a potential TNAP substrate, establishing a possible link between TNAP activity and lipid transport between the liver and the bloodstream.[52] Recently, this group has also shown that *Alpl*$^{-/-}$ mice exhibit liver steatosis and reduced serum triglyceride levels, mirroring the effects of choline deficiency.[17] In addition to choline, hepatocytes can use ethanolamine to produce phosphatidylethanolamine, which is subsequently methylated to form phosphatidylcholine. Although this compensatory pathway cannot fully compensate for severe choline deficiency, it helps sustain phosphocholine production under mild choline scarcity and a TNAP deficiency may disrupt this alternative route of hepatic phosphocholine synthesis. However, an additional or alternative source of PEA may reflect diminished hepatic O-phosphorylethanolamine phospho-lyase activity, the enzyme reported to hydrolyze PEA using PLP as a cofactor.[53] Nevertheless, it is now clear that TNAP hydrolyzes phosphocholine and PEA in the blood. The newly established function of TNAP in choline metabolism is important to consider for individuals with HPP. Treatment with asfotase alfa transitions HPP patients from *hypo*phosphatasemia to *hyper*phosphatasemia, which may alleviate liver choline deficiency. Twenty-two events related to chronic hepatitis were reported in 13/69 (19%) asfotase alfa-treated patients.[54,55] All were mild or moderate in severity, but the origin of these liver complications is unknown and deserves further investigation. The rarity of HPP, combined with its moderate presentation in adult cases, makes it difficult to ascertain whether HPP patients may be more susceptible to developing liver steatosis compared to the general population. Nevertheless, measuring their serum choline levels could reveal subnormal values in HPP patients, perhaps warranting choline supplementation as a prophylactic measure. Given these recent pre-clinical studies linking TNAP function to phosphocholine metabolism in the liver and triglyceride transport from the liver to the bloodstream, it may be of interest to examine the lipoprotein profile in HPP patients at baseline and how that profile may change with enzyme replacement.

Bacterial endotoxins: As reported[56,57] (and references therein), TNAP is an anti-inflammatory nucleotidase, which also has the capacity to dephosphorylate and detoxify bacterial lipopolysaccharide (LPS). LPS is a well-known pro-inflammatory compound synthesized by Gram-negative bacteria, and its pro-inflammatory effects rely on the phosphate groups in the lipid A region. In the blood, TNAP participates in the dephosphorylation of LPS and other bacterial endotoxins.[58] This detoxification role of TNAP may be significant in the understanding of nephrocalcinosis in HPP. The presence of TNAP's activity in the kidney is interesting, since TNAP hydrolyzes PP_i, a potent mineralization inhibitor that is frequently found in hydroxyapatite calculi. Intravenous[32]PP_i is rapidly hydrolyzed in plasma, with PP_i also being filtered at the glomerulus and subject to further hydrolysis within the kidney; only <5% of intravenous[32]PP_i appears in urine. During our pre-clinical studies, we observed that in *Alpl*$^{-/-}$ mice treated with either asfotase alfa or with viral vector delivery of mineral-targeted TNAP (AAV8-TNAP-D_{10}), enzyme replacement prevented the skeletal and dental deficits, accompanied by normalization of plasma PP_i

but urinary PP_i remained abnormally high.[59,60] These data suggest that the largest source of PP_i in the kidney is local generation and that urinary PP_i is a distinct compartment not necessarily influenced by plasma PP_i homeostasis. Why would the HPP kidneys calcify in the presence of high concentrations of urinary PP_i? Besides acting as a pyrophosphatase, TNAP also plays a role as an anti-inflammatory molecule able to progressively dephosphorylate ATP (a pro-inflammatory molecule) to adenosine (an anti-inflammatory molecule) and detoxify bacterial LPS. TNAP itself is upregulated by TNF-α, IL1-β, and IL-6.[57] I suspect that tissue damage caused by inflammation, because of accumulation of ATP and/or bacterial LPS, acts as a nidus for the ectopic deposition of mineral in the kidney parenchyma in HPP that is not overcome by the high urinary PP_i concentrations. It would be worthwhile testing this hypothesis and contrasting it with the more straightforward explanation for nephrocalcinosis as caused by a disturbance of mineral homeostasis.

Phosphorylated osteopontin (OPN) and phosphorylated Tau: Finally, TNAP can also act as a phosphoprotein phosphatase, and at least 2 such phosphoproteins, OPN and Tau, have been shown to behave as natural substrates of TNAP. OPN is a highly phosphorylated glycoprotein, and this phosphorylation is important because phosphorylated, but not dephosphorylated, OPN inhibits mineralization and high plasma levels of phosphorylated OPN accompany the increased extracellular PP_i levels in *Alpl*$^{-/-}$ mice. Ablation of the OPN gene (*Spp1*) in the *Alpl*$^{-/-}$ mice led to partial improvement of the hypomineralization status[61] and we were able to identify at least 2 phosphorylated OPN peptides as targets for TNAP hydrolysis.[62]

It has previously been reported that TNAP dephosphorylates hyperphosphorylated extracellular Tau in the brain, promoting activation of muscarinic receptors.[63] Interestingly, the conditional overexpression of TNAP causes intracellular Tau hyperphosphorylation and aggregation in cells neighboring those overexpressing the ectoenzyme. Conversely, the genetic disruption of TNAP reduced the dephosphorylation of extracellular Tau and decreased neuronal hyperactivity, brain atrophy, and hippocampal neuronal death in a mouse model of tauopathies, decreasing the anxiety-like behavior, motor deficiency, and increasing memory capacity and life expectancy. Similar results were observed by the in vivo pharmacological blunting of TNAP activity using SBI-425.[63] While these pre-clinical studies would seem to point to a protective effect of having low neuronal TNAP activity as far as tauopathies are concerned, there is much to be learned about what central nervous system manifestations might be found in HPP patients.

Current and upcoming treatments

Asfotase alfa/Efzimfotase alfa (mineral-targeted forms of TNAP)

Mineral-targeted TNAP (known as asfotase alfa), is a protein therapeutic designed to prevent and treat the clinical manifestations of HPP. Asfotase alfa consists of the TNAP polypeptide, devoid of its GPI anchoring domain, that was replaced by the human Fc region of immunoglobulin gamma 1 followed by a deca-aspartate acid (D_{10}) sequence to confer mineral-targeting properties due to the high affinity of D_{10} for bone mineral.[59,64] Daily subcutaneous injections of asfotase alfa from birth was shown to prevent infantile HPP disease phenocopied by the *Alpl*$^{-/-}$ mouse model of severe infantile HPP[59], and it also prevented the extensive dental defects, including dentine,[23,65] acellular cementum,[66] and enamel defects.[22] In 2012, asfotase alfa was shown to be effective in infants and young children with life-threatening HPP,[67] and this biologic was approved multinationally for pediatric-onset HPP in 2015 under the name Strensiq. This treatment markedly improves the lifespan, the skeletal phenotype, motor function, and the quality of life of patients with HPP.[67] However, enzyme replacement currently necessitates 3-6 injections of the biologic per week, often leading to injection site reactions severe enough to force discontinuation of treatment[68,69] and it is also very expensive. Efzimfotase alfa (aka ALXN1850) is an investigational second-generation recombinant protein, where a point mutation (E108M) was introduced in the TNAP polypeptide to increase enzymatic activity and 2 of the 5 N-linked glycosylation sites were removed (N213Q and N286Q) to improve the pharmacokinetic properties of the chimeric enzyme.[70] The Fc region was also changed from the human IgG1 to the human IgG2/4 containing hinge, CH2, and CH3 domains. Efzimfotase alfa, appears to have acceptable safety, tolerability, and a pharmacokinetic profile and to achieve higher exposure compared with asfotase alfa and the expectation of a reduced burden of required injections to maintain efficacy. Clinical trials are ongoing.

Viral vector delivery of mineral-targeted TNAP

A collaboration with Professor Shimada's group that started in 2007 at the Hypophosphatasie Europe meeting in Huningue, France, when our first pre-clinical efficacy studies of asfotase alfa were being presented, led to testing the possibility of using viral vectors to deliver mineral-targeted TNAP to prevent/ameliorate HPP. In the subsequent years, several types of viral vectors (lentiviral, adeno-associated serotypes 8 and 9 and self-complementary AAV8), expressing TNAP-D_{10} were shown to prolong life, prevent seizures, and improve the skeletal phenotype of *Alpl*$^{-/-}$ mice after a single injection.[71–73] A refinement of this therapeutic principle was recently achieved using a single intramuscular administration of an AAV8 encoding TNAP-D_{10} to increase the lifespan and improve the skeletal and dentoalveolar phenotypes in *Alpl*$^{-/-}$ mice while obviating the need for the multiple weekly injections in this mouse model of infantile HPP.[60,74] This same vector proved efficacious in ameliorating osteomalacia in the *Alpl*$^{Prx1/Prx1}$ model of late-onset HPP[28] and a model of HPP-like disease caused by PHOSPHO1 deficiency[34] that features early onset scoliosis, reduced plasma TNAP levels, osteomalacia, and dental issues. Investigational new drug-enabling studies have recently been conducted to define an optimal viral dose in both the infantile and later-onset HPP mouse models.[28] Non-human primate studies are currently being conducted by Professor Miyake in Japan in anticipation of initiating clinical trials (submitted for publication).

Because mineral-targeted TNAP was designed to specifically bind to hydroxyapatite, it is inescapable that it also has the potential to bind to sites of ectopic calcification, and we demonstrated that fluorescence-labeled asfotase alfa administered in vivo binds not only to sites of skeletal and dental mineralization but also to mineral in the heart, aorta, and renal artery in animal models displaying severe ectopic

calcification.[75] Our lab has shown that overexpressing TNAP in either the medial[16] or the endothelial[76,77] layers of the vasculature leads to cardiovascular compromise. Given that HPP patients undergoing lifelong treatment with asfotase alfa may develop comorbidities associated with vascular calcification, such as diabetes, CKD, or atherosclerosis, evaluating the consequences of binding of the therapeutic mineral-targeted TNAP to sites of ectopic calcification may be clinically relevant. Results presented during ASBMR 2025 (FRI&SUN 293) showed the consequences of superimposing CKD onto the *Alpl*$^{-/Prx1}$ mouse model of later-onset HPP undergoing long-term treatment with asfotase alfa. We examined skeletal outcomes, renal and vascular calcification following asfotase alfa treatment alone or in combination with CKD induction (submitted for publication). Our findings reveal that the presence of CKD as a comorbidity compromises the efficacy of the ongoing treatment with asfotase alfa and results in aggravated vascular calcification. These data exemplify the need to carefully evaluate treatment dosing and to be observant of extra-skeletal manifestations with time. This also illustrates the need to develop alternative non-mineral-targeted therapeutic strategies for HPP.

Ilofotase alfa (a non-targeted chimeric form of alkaline phosphatase)

We had earlier shown that the sustained availability of soluble (non-targeted) TNAP, introduced via an adeno-associated viral vector[78] also could prolong life, prevent seizures, and improve the skeletal phenotype of *Alpl*$^{-/-}$ mice. This encouraged us to test a chimeric non-targeted form of AP being developed for the treatment of acute kidney injury. This ChimAP (now called Ilofotase alfa) was generated by substituting the flexible crown domain of human intestinal AP with that of the human placental isozyme and has catalytic properties distinct from those of recombinant TNAP, and narrower substrate specificity with selectivity for bacterial-derived LPS compared to the parent intestinal isozyme or to TNAP.[79] Daily injections of 16 mg/Kg/d of ChimAP into *Alpl*$^{-/-}$ mice prolonged life, seizures, and ameliorated skeletal and dental disease[80] although the resolution of osteomalacia was not as profound as that achieved with mineral-targeted TNAP. Ilofotase is currently being evaluated clinically with a focus on normalizing PP_i and PLP and helping with muscle weakness in later-onset HPP patients. Data presented during ASBMR 2025 (1020) showed improvement in the endurance and grip strength of *Alpl*$^{-/Prx1}$ mice.

ENPP1 inhibition

The major producer of PP_i is ectonucleotide pyrophosphatase/phosphodiesterase 1 (ENPP1) which hydrolyzes ATP to AMP and PP_i. ENPP1 deficiency leads to ossification of the posterior longitudinal ligament of the spine, generalized arterial calcification of infancy but can also manifest phenotypic changes characteristic of pseudoxanthoma elasticum that is more typically caused by a deficiency in the ABCC6 transporter.[81] *Enpp1*$^{-/-}$ mice have almost undetectable levels of plasma PP_i leading to inappropriate deposition of hydroxyapatite in soft tissues.[82] Earlier genetic studies by our group demonstrated that the double ablation of the *Alpl* and *Enpp1* genes (*Alpl*$^{-/-}$; *Enpp1*$^{-/-}$ double knockout mice) led to normalization of plasma PP_i concentrations and improvements in skeletal mineralization.[82,83] Thus, we recently tested the potential efficacy of using ENPP1 inhibition as a means of decreasing PP_i concentrations in the *Alpl*$^{-/Prx1}$ mouse model of later-onset HPP. We used the ENPP1 inhibitor REV102 and administered this small molecule admixed to the mouse chow for 105 d followed by X-ray, μCT, and bone morphometry analyses. Our data indicate that the later-onset HPP phenotype can benefit from oral administration of an ENPP1 inhibitor.[84] A particularly interesting finding was the consistently poorly mineralized or plainly absent patella structure in *Alpl*$^{-/Prx1}$ untreated mice, which became clearly recognizable and normal in shape and mineralization in the REV102-treated mice. Could the patella be a useful skeletal site in the clinic to help diagnose or evaluate therapy in adult HPP?

Cell-based therapies

A few studies have attempted cell-based therapy for HPP. The pioneering work of Michael P. Whyte demonstrated improvement in 2 unrelated infants affected by life-threatening HPP using bone marrow (BM) cells as well as cultured osteoblasts together with bone fragments (referenced in[1]). An 8-mo-old girl with infantile HPP underwent cytoreduction and was given T-cell depleted haplo-identical marrow from her sister. Engraftment was achieved, but she needed marrow enriched with mesenchymal stems cells after 6 mo. Prolonged improvement of her skeletal condition was observed, but she remained small. The second child, a 9-mo-old girl with infantile HPP received a combination of BM, engrafted bone fragments from the father (sc and ip) and osteoblasts from the bone fragments. Four months later, radiographs demonstrated improved skeletal mineralization. This patient, 7 yr after transplantation, was active and growing, and displayed the clinical phenotype of the milder, childhood form of HPP. Subsequently, 2 patients (an 8-mo-old boy and a 14-mo old girl) with severe HPP underwent BM transplantation after myeloablative conditioning followed by multiple infusions of allogeneic mesenchymal stem cells expanded ex vivo.[85] The donors were asymptomatic relatives harboring heterozygous variants of the *ALPL* gene. There were improvements in bone mineralization, muscle mass, respiratory function, and mental development, resulting in the patients being alive at the age of 3. However, restoration of AP activity was limited, and normal bony architecture could not be achieved.

There is also some documented experience of cell-based therapies using the *Alpl*$^{-/-}$ model of infantile HPP using lentivirally transduced BM cells expressing TNAP-D_{10}.[86] The treated *Alpl*$^{-/-}$ mice were normal in appearance and experienced no seizures during the experimental period. The transduced cells efficiently engrafted and supplied TNAP-D_{10} (400-fold serum AP compared to mock-treated mice) continuously at a therapeutic level for at least 3 mo. Moreover, TNAP-D_{10} overexpression did not affect multilineage reconstitution in the recipient mice. The plasma AP activity was sustained at high levels in the treated mice, and tissue AP activity was selectively detected on bone surfaces, not in the kidneys or other organs. No ectopic calcification was identified. More recently, another lentiviral vector was used to stably express soluble TNAP in transduced hematopoietic stem/progenitor cells. Engraftment and differentiation of these transduced cells showed that the cell therapy approach led to a durable correction of plasma AP activity, rescued skeletal manifestations, and prevented early mortality in the *Alpl*$^{-/-}$ mice (submitted for publication).

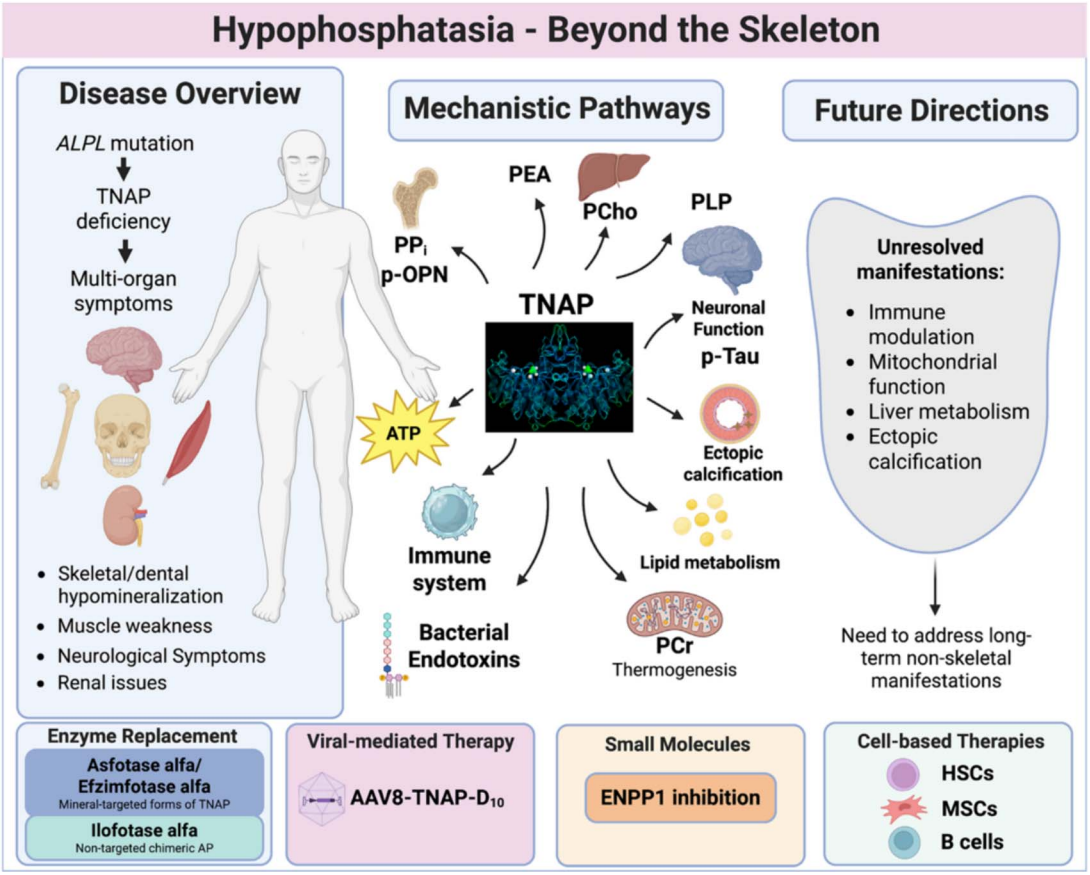

Figure 1. Graphical representation of the contents of this invited perspective article reflective of the topics discussed during the Louis V. Avioli Memorial Lecture delivered during ASBMR 2025. The panels summarize the brief introduction of the disease hypophosphatasia (HPP) and its genetic basis, followed by the major focus of this article on describing the natural substrates of tissue-nonspecific alkaline phosphatase and the biochemical pathways that may be affected in HPP as understood primarily from preclinical studies employing mouse models of HPP. Finally, a discussion of some unresolved manifestations in HPP and an update on the currently approved treatment with asfotase alfa and upcoming therapeutic approaches that are at different stages of development.

Novel phenotypic changes uncovered in the mouse models of HPP

Immune system abnormalities are frequently reported in HPP patients. Recurrent chest, sinus, and ear infections, longer times to recover from illness, persistent fevers, and abnormal responses to immunizations are some of the symptoms described by patients, suggesting a dysfunction of the immune system.[87] HPP patients experience inflammatory conditions, such as BM edema/chronic non-bacterial osteomyelitis, myopathies, tendinitis, and increased predisposition for periodontitis.[88] An exploratory study revealed that bone metaphyses of 7-d-old *Alpl*$^{+/-}$ mice had significantly increased levels of IL-1β and IL-6 and decreased levels of the anti-inflammatory IL-10 cytokine compared to Wt mice.[56] TNAP increases during inflammation and participates in the modulation of inflammatory responses via hydrolyzing pro-inflammatory ATP and increasing the concentrations of anti-inflammatory adenosine.[37,56,88] TNAP expression has been reported in different types of immune cells, including neutrophils, T cells, and B cells. Results presented during ASBMR 2025 (1017) showed that while the total number of B cells and B cell progenitors are unchanged in 6-wk-old AAV8-TNAP-D_{10} treated *Alpl*$^{-/-}$ mice, pre-pro B cells are significantly reduced. Conversely, these cell populations were increased in mice where TNAP was overexpressed in every cell type. These findings indicate that TNAP is involved in B cell development. This study also demonstrates that pharmacological inhibition of TNAP with SBI-425 reduces the expression of activation and differentiation markers in B cells stimulated in vitro. These data point to an involvement of TNAP in B cell activation and differentiation to antibody secreting plasma cells. Overall, these findings support the importance of TNAP in B cell development and expose its role in B cell function and indicate that there are intrinsic B cell defects in HPP mice, which are not preventable by the treatment with mineral-targeted TNAP and thus point to the need to evaluate immune cell function in HPP patients undergoing life-long treatment with asfotase alfa. A similar caveat is applicable to the potential use of ENPP1 inhibition. Given that another known function of ENPP1 is the hydrolysis of cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) that activates the Stimulator of Interferon Genes (STING) pathway,[89] future studies should focus on assessing any deleterious or beneficial effects of modulating the STING pathway in the context of HPP. Considering the immunoregulatory role of OPN,[90] and that TNAP affects its function via dephosphorylation, the TNAP-OPN axis in the immune system is another area worthy of investigation.

Concluding remarks

Figure 1 graphically lays out the topics discussed in this invited perspective article that follows the contents of the Louis V. Avioli Memorial lecture delivered during the ASBMR

2025 annual meeting. While the principal clinical signs, symptoms, and complications of HPP are related to defective bone and tooth mineralization, severely affected patients can now survive their disease thanks to lifesaving asfotase alfa but there remain also milder pediatric and adult forms of HPP that also cause suffering and for which we do not have an approved therapy. As novel therapies are conceived and implemented, it is crucial to recognize what aspects of HPP symptomatology we are not yet improving to better address those manifestations, and to be cognizant of the potential consequences of turning certain cell types and organs into factories for a therapeutic product (such as in gene-editing strategies engaging the immune system or the liver). Understanding what physiological role TNAP has in those organs can help with the rational design of improved therapies.

Author contributions

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Conflicts of interest

The author reports no conflicts of interest.

Data availability

No experimental data are presented in this perspective article. The data referenced here are available in the respective published or soon to be published articles.

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Journal of Bone and Mineral Research, 2026, 41, 208–219
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