Actual. Osteol 8(3): 164-182, 2012.
Internet: http://www.osteologia.org.ar

ACTUALIZACIONES / *Reviews*

HYPOPHOSPHATASIA - PATHOPHYSIOLOGY AND TREATMENT

José Luis Millán[1], Horacio Plotkin[2]

1) Sanford Children's Health Research center, Sanford-Burnham Medical Research Institute, La Jolla, CA 92037. 2) Alexion Pharmaceuticals, Cambridge, MA 02142.

Summary

Hypophosphatasia (HPP) is the "inborn-error-of-metabolism" caused by loss-of-function mutation(s) in the gene that encodes the tissue-nonspecific isozyme of alkaline phosphatase (TNAP). The disease can be classified according to patient age when the first signs and symptoms manifest; i.e., perinatal, infantile, childhood, adult HPP. Odonto HPP presents with only dental problems. Babies with the perinatal/infantile forms of HPP often die with severe rickets and sometimes hypercalcemia and vitamin B_6-responsive seizures. The primary biochemical defect in HPP is a deficiency of TNAP catalytic activity that leads to elevated circulating levels of inorganic pyrophosphate (PP_i), a potent calcification inhibitor. To-date, the management of HPP has been essentially symptomatic or orthopedic. However, enzyme replacement therapy with mineral targeting TNAP (sALP-FcD10, a.k.a. or asfotase alfa) has shown remarkably successful results in a mouse model of HPP ($Alpl^{-/-}$ mice). Administration of mineral-targeting TNAP from birth increased survival and prevented the seizures, rickets, as well as all the tooth abnormalities, including dentin, acelular cementum, and enamel defects, in this model of severe HPP. Clinical trials using mineral-targeted TNAP in children 3 years of age or younger with life-threatening HPP was associated with healing of the skeletal manifestations of HPP as well as improved respiratory and motor function. Improvement is still being observed in the patients receiving continued asfotase alfa therapy, with more than 3 years of treatment in some children. Asfotase alfa appears to be a potential enzyme replacement therapy in patients with life-threatening HPP.

Keywords: *Hypophosphatasia, alkaline phosphatase, asfotase alfa.*

** Dirección postal: José Luis Millán, Ph. D. Sanford-Burnham Medical Research Institute 10901 North Torrey Pines Road. La Jolla, CA 92037. Correo electrónico: millan@sanfordburnham.org*

Resumen

HIPOFOSFATASIA – FISIOPATOLOGÍA Y TRATAMIENTO

La hipofosfatasia (HPP) es un error innato del metabolismo causado por mutaciones con pérdida de función en los genes que codifican la fosfatasa alcalina tejido no específica (TNAP). Esta enfermedad puede clasificarse de acuerdo a la edad en la que los primeros signos son aparentes (perinatal, infantil, de la niñez y adulta). Un tipo particular es la odontofosfatasia, en la que solo el cemento dental está afectado, sin manifestaciones óseas. Pacientes con las formas perinatal e infantil frecuentemente fallecen con raquitismo severo, hipercalcemia, y, en ocasiones, convulsiones que responden al tratamiento con vitamina B_6. El defecto bioquímico primario en HPP es una deficiencia de actividad catalítica de TNAP, que conlleva a una elevación de los niveles séricos de pirofosfato inorgánico (PPi), que es un potente inhibidor de la calcificación. Hasta ahora, el manejo de la HPP ha sido sintomático y/u ortopédico. Sin embargo, el uso de una terapia de reemplazo enzimático con TNAP modificada para ser más ávida por el tejido óseo (sALP-FcD_{10}, también llamada *ENB-0040 o* asfotasa alfa) ha mostrado resultados remarcables en un modelo murino de HPP (ratón *Alpl*$^{-/-}$). La administración de TNAP dirigida específicamente al mineral óseo aumentó la supervivencia y previno convulsiones, raquitismo y anomalías dentarias (incluyendo dentina, cemento acelular y defectos del esmalte) en este modelo de HPP severa. Estudios de investigación clínica utilizando TNAP dirigida específicamente al mineral óseo en niños menores de 3 años con HPP potencialmente letal demostraron que su uso se asocia con resolución de las manifestaciones óseas de HPP, así como con mejoría en la función motora y respiratoria. Los pacientes continúan respondiendo aún después de más de tres años de tratamiento con asfotasa alfa. El tratamiento de HPP con asfotasa alfa es un tratamiento prometedor para pacientes con HPP potencialmente letal.

Palabras claves: hipofosfatasia, fosfatasa alcalina, asfotasa alfa.

The disease

Hypophosphatasia (HPP) is a rare, heritable form of rickets or osteomalacia with an incidence for the severe forms ranging from 1:100,000 in the general population[1] to as great as 1 per 2,500 births in Canadian Mennonites.[2] This "inborn error of metabolism" is caused by loss-of-function mutation(s) in the chromosome 1 gene (*ALPL*) that encodes the tissue-nonspecific isozyme of alkaline phosphatase (TNAP; a.k.a. liver/bone/kidney type AP). The other three isozymes, i.e., placental AP (PLAP), placental-like or germ cell AP (GCAP), and intestinal AP (IAP), all syntenic in chromosome 2, are encoded by *ALPP*, *ALPP2*, and *ALPI*, respectively, and have a much more restricted, i.e. tissue-specific, expression pattern.[3]

The clinical severity of HPP varies greatly. The disease can be classified according to patient age when the first signs and symptoms manifest; i.e., perinatal, infantile, childhood, and adult HPP. Additional clinical forms include odonto-HPP where there is only dental manifestations and prenatal benign-HPP.[4] The severity of HPP ranges from absence of bone mineralization and stillbirth to only dental problems with no bone phenotype. Perinatal (lethal) HPP is seen in utero and can cause stillbirth.[5] Some of these neonates may survive several days but suffer increased respiratory compromise due to the hypoplastic and rachitic deformity of the chest. Common features are a failure to gain weight, irritability, high-pitched cry, fever of unknown origin, periodic apnea, myelophthisic anemia, intracranial hemorrhage, and vitamin B_6-responsive seizures. Infantile HPP presents before 6 months of age. Postnatal development often appears normal

until the onset of poor feeding, inadequate weight gain, and rickets. The marked radiological features are characteristic and sometimes progressive, and resemble those found in the perinatal form although somewhat less severe. Serial radiological studies may reveal persistence of impaired skeletal mineralization as well as gradual demineralization of osseous tissue. Childhood HPP has also highly variable clinical expression. Premature loss of deciduous teeth results from aplasia, hypoplasia or dysplasia of dental cementum that connects the tooth root with the periodontal ligament. Rickets causes short stature and the skeletal deformities may include bowed legs, enlargement of the wrists, knees, and ankles as a result of widened metaphyses. Morbidity can be significant, including compromise of ambulation and activities of daily living. Adult HPP usually presents during middle age, although frequently there is a history of rickets and/or early loss of teeth followed by good health during adolescence and young adult life.[6] Then, recurrent metatarsal stress fractures are common and calcium pyrophosphate dihydrate deposition can cause attacks of arthritis and pyrophosphate arthropathy.[7] Again, morbidity can be significant, with numerous fractures, chronic pain, and loss of ambulation. Odonto HPP is diagnosed when the only clinical abnormality accompanying biochemical and genetic alterations is dental disease and radiological studies and even bone biopsies reveal no signs of rickets or osteomalacia. Prenatal benign HPP is suspected when patients present with limb deformities in-utero or at birth, but without chest deformities or respiratory compromise.[8] The subjects then show spontaneous improvement in their deformities.

A multitude of TNAP mutations leads to suboptimal activity of TNAP that in turn leads to HPP. The first identified HPP mutation was a homozygous missense mutation in exon 6 of the *ALPL* gene, A162T.[9] Subsequently, compound heterozygosity and new mutations, (i.e., R54C, R54P, E174K, Q190P, Y246H, D277A, D361V, and Y419H) were reported.[10, 11] Other mutations were then described such as G317D[2]; E281K, A160T, F310L and G439R[12] and a frame shift-mutation at position 328 and another at position 503.[13] Mornet *et al.* (1998) reported 16 new missense mutations in European patients (i.e., S-1F, A23V, R58S, G103R, G112R, N153D, R167W, R206W, W253X, E274K, S428P, R433C, G456S, G474R and splice mutations in intron 6 and 9).[14] Interestingly, in a historical vignette, the mutations present in the original 3-week-old infant boy with HPP reported by Rathbun in 1948 were identified.[15] That patient was a compound heterozygote for the A97T and D277A mutations. Dr. Etienne Mornet has compiled a downloadable database of all known TNAP mutations and polymorphisms which lists ~ 260 distinct mutations (http://www.sesep.uvsq.fr/Database.html).

Pathophysiology of HPP

The primary biochemical defect in HPP is a deficiency of TNAP catalytic activity which leads to elevated circulating levels of inorganic pyrophosphate (PP_i), pyridoxal-5'-phosphate (PLP), and phosphoethanolamine (PEA) which are all molecules that are presumed to be substrates of TNAP. PP_i and PEA also appear in the urine at increased levels and can be used as diagnostic markers.

In skeletal tissues, TNAP is confined to the cell surface of osteoblasts and chondrocytes, including the membranes of their shed matrix vesicles (MVs)[16, 17] where the enzyme is particularly enriched.[18] Electron microscopy revealed that TNAP-deficient MVs, in both humans and mice, contain hydroxyapatite crystals, but that extravesicular crystal propagation is retarded.[19-21] These abnormalities are caused by the extracellular accumulation of PP_i, a potent inhibitor of calcification[22], in the bone matrix due to absent TNAP pyrophosphatase activity. This suggests that PP_i

is a natural substrate of TNAP.[23-25] Breeding *Alpl*$^{-/-}$ mice to mice deficient in the production (*Enpp1*$^{-/-}$) or transport (*ank/ank*) of PP_i partially corrects the skeletal defect in *Alpl*$^{-/-}$ mice, confirming that increased PP_i levels are the culprit causing rickets/osteomalacia in HPP.[23, 24]

We observed previously that ATP-dependent[45] Ca precipitation was reduced in calvarial osteoblast-derived MVs isolated from *Alpl*$^{-/-}$ mice[26], an observation that we had attributed to the increases in MV PP_i content resulting from reduced PP_i hydrolysis in *Alpl*$^{-/-}$ MVs. However, Ciancaglini *et al.* reported that TNAP is not only an efficient PP_iase but that in the MVs compartment it is also a potent ATPase and ADPase.[27] Thus, our data support the two functions that had been proposed for TNAP during skeletal mineralization: i) a P_i-generating enzyme, and ii) an enzyme that destroys a potent inhibitor of mineralization.[5, 28-31] Hence, TNAP seems to play a major role as a pyrophosphatase promoting extravesicular growth of bone mineral while it generates P_i from ATP and ADP in the perivesicular space to help initiate intravesicular mineralization.[32]

Another substrate of TNAP is pyridoxal-5'-phosphate (PLP), the predominant circulating vitameric form of Vitamin B_6. Vitamin B_6 serves as a cofactor for at least 110 enzymes, and can be found in three free forms (or vitamers), i.e., pyridoxal (PL), pyridoxamine (PM), and pyridoxine (PN), all of which can be phosphorylated to the corresponding 5'-phosphated derivatives, PLP, PMP, and PNP.[33, 34] PLP serves as a coenzyme in reactions that involve the catabolism of various amino acids and decarboxylation reactions necessary for generation of the neurotransmitters dopamine, serotonin, histamine, γ-aminobutyric acid (GABA), and taurine. The phosphorylation of PL, PM, and PN is catalyzed by PL kinase. PLP and PMP are interconvertible through aminotransferases or PMP/pyridoxine 5'-phosphate oxidase. Removal of the phosphate group is a function of TNAP.[35] Since only dephosphorylated vitamers can be transported into the cells, decreased TNAP activity in HPP results in marked increases in plasma PLP levels.[35-38] Injection or ingestion of PL or PM, both hydrophobic forms of vitamin B_6 that can easily traverse biological membranes, temporarily suppresses the epileptic seizures in *Alpl*$^{-/-}$ mice confirming the role of TNAP in the metabolism of PLP in vivo.[39-41] However, *Alpl*$^{-/-}$ mice still have a 100% mortality rate at weaning, and their demise is often heralded by epileptic seizures.

The origin of the increased urinary excretion of PEA in HPP remains unclear. One possibility is that PEA is a natural substrate of TNAP[35], but the putative metabolic pathway involved has not been elucidated. An alternative explanation relates to abnormalities in the function of O-phosphorylethanolamine phospholyase (PEA-P-lyase), an enzyme reported to require PLP as cofactor.[42, 43] Since TNAP is crucial for the hydrolysis of PLP, possibly insufficient PLP inside cells leads to suboptimal activity of PEA-P-lyase which in turn increases excretion of PEA in HPP. Of interest in this regard, members of a large kindred with adult HPP showed PEA and phosphoserine levels in their urine, which correlated inversely with both total and liver TNAP activity in their serum but not with the activity of bone TNAP.[44]

Alpl$^{-/-}$ mice also display marked changes in osteopontin (OPN, encoded by Spp1) levels, with elevated expression at both its RNA and protein levels.[45-47] While the biological role of OPN is incompletely understood, one known function is to anchor osteoclasts to the hydroxyapatite surface through its poly-aspartic acid sequences, while it also binds to CD44 and $\alpha_v\beta_3$ integrin via its RGD sequence and mediates cell signaling and/or migration.[48] OPN is a highly phosphorylated glycoprotein, with 36 serine/threonine phosphorylation sites in the human protein.[49] This phosphorylation is

functionally important as the inhibitory effect of OPN on mineral deposition was diminished if 84% of the covalently bound phosphates were removed from OPN.[50] Phosphorylated OPN inhibits mineralization in vascular smooth muscle cells, while dephosphorylated OPN does not.[51] Certain phosphorylated OPN peptides are also capable of inhibiting hydroxyapatite formation in vitro[52] and cause dose-dependent inhibition of mineralization in cultured cells.[53] OPN expression is controlled by PP_i levels [45, 53] and high plasma OPN levels accompany the increased extracellular PP_i levels in *Alpl*$^{-/-}$ mice. In turn, [*Alpl*$^{-/-}$; *Spp1*$^{-/-}$] double knockout mice, have partial improvement of the hypomineralization of *Alpl*$^{-/-}$ mice.[45] This indicates that OPN accumulation contributes to the impaired bone mineralization of *Alpl*$^{-/-}$ mice.[45] Recently, the Millán laboratory used genetic means to alter the phosphorylation status of OPN *in vivo*, and showed that absence of TNAP function leads to accumulation of phosphorylated OPN (Narisawa *et al.*, unpublished results). This defines OPN as another natural substrate of TNAP, and points to alterations in OPN phosphorylation as an additional biochemical pathways involved in the pathophysiology of HPP (at least in mice). Future studies will need to look at OPN status in patients with HPP.

To summarize our current views of the pathophysiology of HPP as revealed by the *Alpl*$^{-/-}$ mouse model which recapitulates the infantile form of HPP: lack of TNAP activity leads to reduced perivesicular production of P_i, extravesicular accumulation of PP_i and accumulation of phosphorylated OPN, all factors that cause soft bones (rickets/osteomalacia). Inadequate extracellular levels of PL, due to insufficient dephosphorylation of PLP, causes the epileptic seizures that are 100% penetrant in this null TNAP model.

Clinical management of HPP to-date

To-date, the management of HPP has been essentially symptomatic or orthopedic.[4] For example, infants with hypercalcemia from HPP may require restriction of dietary calcium or administration of calciuretics, or pediatric patients with craniosynostosis may need surgical intervention. Likewise, fractures are treated by prolonged casting or stabilization with orthopedic hardware while dental hygiene should be carefully monitored in individuals with dental manifestations. Until recently, attempts at more definitive HPP treatment have met with limited success. Use of a bisphosphonate (a synthetic analog of pyrophosphate) in one infant reportedly had no discernible effect on the skeleton, and the infant experienced progressive disease with death at 14 months of age.[54] In 2012, aminobisphosphonate treatment for "osteoporosis" may have unmasked HPP in an adult.[55] This is not surprising, because bisphosphonates are synthetic analogs of pyrophosphate. Marrow cell transplantation has been attempted for two severely affected infants and was followed by some clinical and radiographic improvement, but significant morbidity remains.[56, 57]

Anabolic treatment with parathyroid hormone (1-34 or 1-84) apparently benefitted some adults with HPP [58-61]; however, others have reported no benefit.[62, 63] This agent is not approved for children because its use was associated with osteosarcoma in rats.[64] Patents have been filed relevant to an anti-sclerostin agent developed primarily to treat osteoporosis as a means of promoting osteoblastic activity and thereby stimulating TNAP activity in HPP patients (Patent # WO 2008/092894 A1, published Aug 7, 2008: Modulators of sclerostin binding partners for treating bone related disorders), but no data concerning this treatment for HPP has been published. The Millán laboratory is also developing small molecule activators of TNAP activity that could potentially be used to upregulate residual TNAP activity seen in some HPP patients.[65] However, all of these anabolic treatments, includ-

ing parathyroid hormone, anti-sclerostin and TNAP activators, presume a sufficiently mild *ALPL* mutation(s) such that the enzyme retains sufficient catalytic activity for upregulation. Such a circumstance may apply to a subset of HPP patients, presumably those with milder, adult forms of the disease but will therefore not likely be effective in more severe forms of the disease.

It would seem intuitive that providing active TNAP to HPP patients should lead to a decrease in extracellular PP_i levels that would result in improved skeletal mineralization. However, the early trials of enzyme replacement therapy (EzRT) using intravenous infusions of plasma, for example from Paget bone disease patients that contains high TNAP activity, were disappointing.[66, 67] Whyte and colleagues tested EzRT for a 6-month-old girl with infantile HPP[66] by administering repeated intravenous infusions of TNAP-rich plasma obtained by plasmapheresis from two men with Paget bone disease. Her circulating AP activity had a half-life of ~ two days, similar to that reported in adults, which did not change during a five-week period of six AP infusions. Sequential radiographic studies revealed an arrest of worsening rickets with slight remineralization of metaphyses, although urinary levels of PEA and PP_i were unaltered. This approach was used for three additional patients with infantile HPP[67] who received similar weekly intravenous infusions. Despite partial or complete correction of the deficiency of circulating AP activity, they observed no radiographic evidence for arrest of progressive osteopenia or improvement in rachitic defects in any of the patients and concluded that the failure of infants with HPP to show significant healing of rickets on correction of circulating TNAP activity supported the hypothesis that TNAP functions *in situ* during skeletal mineralization. In a similar study, Weninger and colleagues attempted EzRT for a severely affected premature boy with HPP by infusions of purified human liver TNAP. Sequential radiographic studies showed no improvement of bone mineralization.[68]

From these studies, it would appear that TNAP activity must be increased not in the circulation, but in the skeleton itself to prevent or reverse the pathophysiology of HPP. This hypothesis is supported by the improvement demonstrated by two unrelated girls with infantile HPP following attempts to transplant healthy mesenchyme-derived cells where perhaps small numbers of TNAP-containing osteoblasts and chondrocytes were introduced throughout the skeleton.[57, 58] Below, we describe the recent therapeutic breakthrough achieved by targeting TNAP to mineralizing tissues.

Targeting TNAP to bone mineral

In 2005, Enobia Pharma, Montreal, Canada bio-engineered and expressed mineral-targeting recombinant TNAP in CHO cells by modifying the coding sequence of human TNAP in several ways[69]: firstly, the GPI anchor sequence of the hydrophobic C-terminal domain of human TNAP was removed to generate a soluble, secreted enzyme (sALP); secondly, the human TNAP ectodomain sequence was extended with the coding sequence for the Fc region of human IgG gamma1 (Fc); and finally the C-terminus of the Fc region was extended with ten aspartate residues (D_{10}) because this acidic oligopeptide moiety had previously been demonstrated to be an effective carrier for selective drug delivery to bone.[70-72] The resulting fusion protein comprised of 726-amino acids was initially designated sALP-FcD_{10} to indicate the different domains that had been fused together. Subsequently, it was renamed ENB-0040 when it was produced under Current Good Manufacturing Practice (cGMP), and finally renamed asfotase alfa, soon after this protein reached clinical trials in HPP patients. This

is also the name adopted by Alexion Pharmaceuticals, Cheshire, CT who acquired Enobia Pharma in 2012. Here, we retain all three designations to precisely recount which studies where done with what batch of this recombinant mineral-targeted TNAP. While the Fc region was incorporated into ENB-0040 primarily to facilitate purification, Fc is also known to considerably increase the circulating half-life of short-lived proteins, thus allowing lower doses and less frequent injections (Fig. 1). The pharmacokinetics (PK) and tissue distribution properties of sALP-FcD$_{10}$ were determined in studies of adult and newborn mice comparing different administration routes.[69] Figure 1 shows the histochemical staining for ALP activity in untreated and treated *Alpl*$^{-/-}$ mouse bone sections, which proved the localization and activity of injected sALP-FcD$_{10}$ in bone tissue.

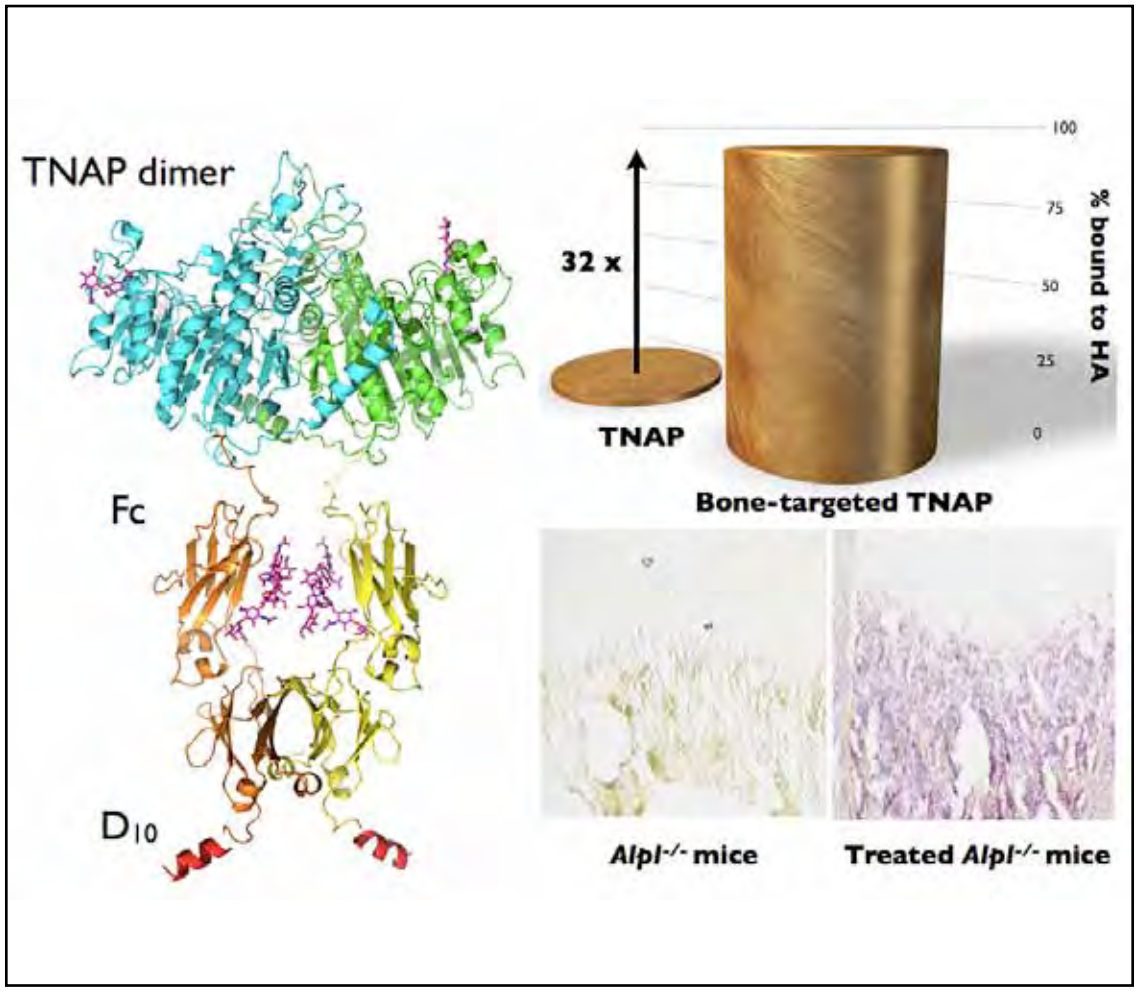

Figure 1. *Left panel:* three-dimensional modeling of ENB-0040. The model shows rigid AP and Fc modules connected by a highly flexible linker. The terminal poly-Asp region is exposed on the opposite site of the AP module. The whole structure is dimeric that conforms to the preferred oligomeric state of the AP as well as the Fc region of the antibody. The three active site metal ions (two Zn^{2+} and one Mg^{2+}) are marked with blue spheres. *Upper right panel:* The affinity of the purified ENB-0040 for hydroxyapatite mineral was compared to that of soluble TNAP purified from bovine kidney. ENB-0040 bound 32-fold more efficiently to reconstituted hydroxyapatite (HA) than did unmodified bovine kidney TNAP (Data taken from Millán *et al.*[69]) *Lower right panel:* Histochemical staining for ALP activity in long bones of an ENB-0040–treated *Alpl*$^{-/-}$ mouse compared with an age-matched untreated *Alpl*$^{-/-}$ mouse.

Preclinical validation of EzRT for HPP using recombinant mineral-targeting TNAP

Alpl$^{-/-}$ mice, which were created via homologous recombination by insertion of the Neo cassette into exon 6 of the mouse gene encoding TNAP (*Alpl*, a.k.a. *Akp2*),[39] show no detectable TNAP mRNA or protein. Phenotypically, *Alpl*$^{-/-}$ knockout mice closely mimic infantile HPP.[73] Like HPP patients, *Alpl*$^{-/-}$ mice exhibit global deficiency in TNAP activity, endogenous accumulation of the ALP substrates PP_i and PLP, and postnatally acquire impaired mineralization of skeletal matrix leading to rickets or osteomalacia. *Alpl*$^{-/-}$ mice manifest stunted growth, and develop radiographically and histologically apparent rickets together with epileptic seizures and apnea, and die between postnatal days 10-12.[39, 41, 73]

The first disease efficacy study utilized subcutaneous sALP-FcD_{10} injections of newborn *Alpl*$^{-/-}$ mice daily for 15 days at 1 mg/kg per dose.[69] µCT analysis of animals treated at this dosage showed no attenuation of skeletal disease in the calvarium, and the proximal tibial growth plate (physis) showed excessive widening of the hypertrophic zone, consistent with early rickets in both sALP-FcD_{10}- and vehicle-injected *Alpl*$^{-/-}$ animals. Increasing the daily dose to 2 mg/kg improved the general appearance, body weight, and tail length of treated *Alpl*$^{-/-}$ mice, which also showed a normal growth rate. Next, *Alpl*$^{-/-}$ mice underwent 15 days of daily SC injections at 8.2 mg/kg sALP-FcD_{10}. Treated animals had greater body weight than vehicle-treated mice, and had plasma PP_i concentrations that were in the normal range. In this short-term experiment, sALP-FcD_{10} treatment minimized hypomineralization in the feet and reduced the number of mice that exhibited severely dysmorphic rib cages. Similarly, the hind limbs appeared healthy in all treated animals.[69] Long-term (52 day) survival and complete prevention of skeletal defects and epilepsy were obtained with daily subcutaneous injections of 8.2 mg/kg sALP-FcD_{10}, an outcome accompanied by normal plasma pyridoxal concentrations and unremarkable calcium concentrations.

In these mice, sALP-FcD_{10} treatment also prevented hypomineralization of alveolar bone and dentin[69] and acellular cementum formed normally, which is typically missing or reduced in *Alpl*$^{-/-}$ mice.[74] Micro-computed tomography revealed a consistent reduction of mineralization in both alveolar bone and root dentin (and root analogue dentin in the incisor) in the control (vehicle-alone-injected) *Alpl*$^{-/-}$ mice (Fig. 2). This feature was not present in the sALP-FcD_{10}-treated *Alpl*$^{-/-}$ mice, which showed complete mineralization of alveolar bone and dentin. Immunohistochemical staining for OPN (Fig. 2), revealed absence of acellular cementum along the root surface in untreated *Alpl*$^{-/-}$ mice, while sALP-FcD_{10}-treated *Alpl*$^{-/-}$ mice had an unremarkable acellular cementum layer comparable with that seen in wild-type mice.[74] More recently, we showed that the dentin defect is root-targeted and varies from delayed mineralization in the mildest case, to arrest of root mantle dentin mineralization, lack of circumpulpal dentin, and odontoblast differentiation defects, in the most severe manifestations.[75] The mildly delayed mineralization was corrected given time, but when dentinogenesis was significantly perturbed at the mantle dentin phase, defects persevered throughout molar tooth development. Our data further reveal that the *Alpl*$^{-/-}$ dentin defect resulted from inability of MV-initiated mineralization foci to expand into a mineralization front, and that increased OPN accumulation likely contributed to the aborted mineralization of *Alpl*$^{-/-}$ mantle dentin. Importantly, administration of bioengineered TNAP showed that early intervention allows for rescue of dentinogenesis and restoration of molar mineralization.[75]

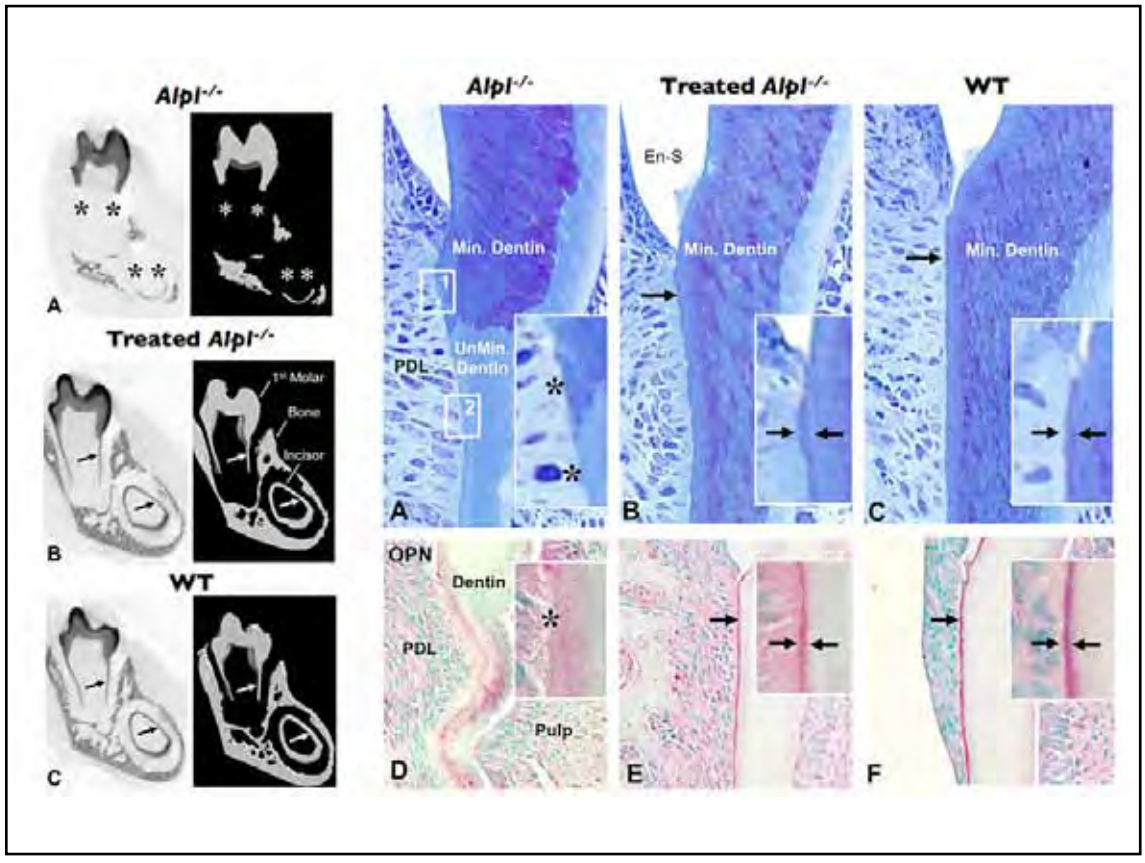

Figure 2. *Left panels:* Targeting TNAP to mineral restores bone and tooth mineralization defects seen in TNAP-deficient (*Alpl*$^{-/-}$) mice, as assessed by micro-computed tomography. (A) Vehicle-injected *Alpl*$^{-/-}$ mice show extensive regions of unmineralized root (and root analogue) dentin (asterisks), as well as surrounding hypomineralized alveolar bone. (B) ENB-0040-treated *Alpl*$^{-/-}$ mice show complete mineralization of all incisor and molar tooth tissues and alveolar bone, which are indistinguishable from WT tissues (C). Arrows indicate mineralized dentin and acellular cementum combined defects. *Right panels:* Presence of acellular cementum in ENB-0040-treated *Alpl*$^{-/-}$ mice visualized by histology and immunohistochemistry with anti-osteopontin antibodies. Tissues appear comparable to those seen in WT mice (arrows and insets). The acellular cementum layer is absent (asterisks) in vehicle-injected *Alpl*$^{-/-}$ mice. Rectangles 1 and 2 in A indicate sites adjacent to mineralized (Min.) and unmineralized (UnMin.) dentin, respectively. Unmineralized dentin is commonly seen in TNAP-deficient mice. Electron micrographs of boxed areas 1 and 2 are shown in Fig. 2. (D-F) Immunohistochemical localization of osteopontin (red, arrows and inset), as a marker of acellular cementum, confirms histologic observations in corresponding panels A-C showing acellular cementum following ENB-0040 treatment (*Alpl*$^{-/-}$-treated) but absence of a discrete immunostained layer (asterisk in inset) in vehicle-treated *Alpl*$^{-/-}$ mice. PDL, periodontal ligament; En-S, enamel space after decalcification. Magnification bars equal 100 µm. Figures and legends taken from McKee *et al.*[74] and reproduced with permission from the *Journal of Dental Research.* OPN: osteopontin.

A subsequent preclinical study using *Alpl*$^{-/-}$ mice defined the dose/response relationship between increasing amounts of cGMP-produced ENB-0040 given by bolus subcutaneous injections and the therapeutic response after 43 days, in anticipation of clinical trials.[76] End-points were survival, body weight, bone length of the tibiae and femora, and bone mineralization defects, as assessed using radiographs of the feet, rib cage, and lower limbs. To date, these parameters have served as accurate indicators of correction of the HPP phenotype in

this animal model. Also, radiographic manifestations of HPP are readily observed in affected infants and are thus represent an important endpoint in preclinical proof-of-concept studies to extrapolate from mice to humans. In addition, we used μCT (Fig. 3) and histomorphometric analysis to evaluate improvement in *Alpl*$^{-/-}$ bone mineralization status for representative age groups undergoing treatment. We documented a clear relationship between daily ENB-0040 dose and the percentage of mice with normal bony structures of the foot, rib cage, and lower limbs. We focused on establishing an effective dose in 80% of the mice (ED_{80}). In mice, that dose was ~ 3.2 mg/kg/day in order to see improvement for the feet, ~ 2.8 mg/kg.day for rib cage, and ~ 2.9 mg/kg.day for lower limbs. These ED_{80} doses were consistent with our previous experience showing enhanced skeletal integrity following short-term, 15-day treatment of *Alpl*$^{-/-}$ animals using 2 mg/kg.day of ENB-0040.[69] The ED_{80}, along with enzyme concentrations measured in animal efficacy studies and ENB-0040 PK data were used to estimate the minimum effective concentrations for *Alpl*$^{-/-}$ mice.

Recent *Alpl*$^{-/-}$ mouse studies have continued to validate the usefulness of mineral targeting TNAP to prevent HPP abnormalities, at sites

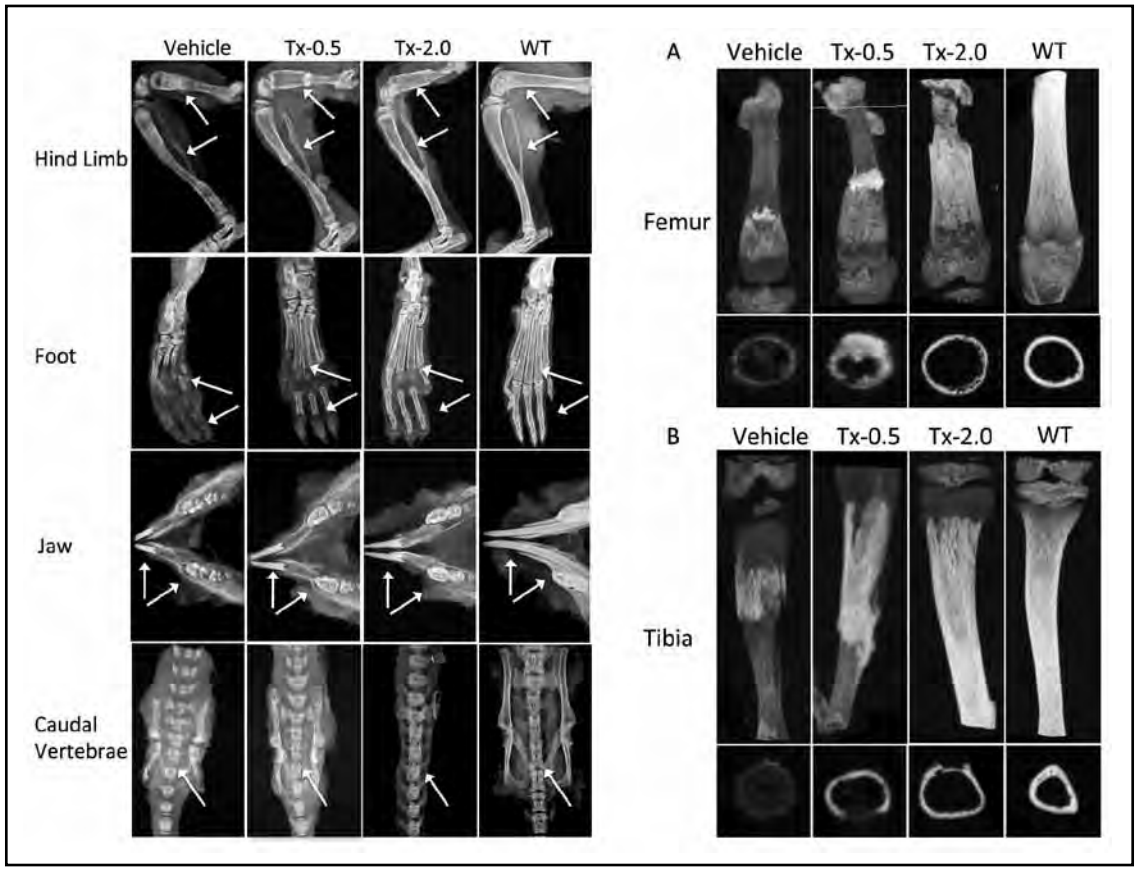

Figure 3. *Left panels:* Representative radiographs of hind limb, foot, jaw bones and caudal vertebrae specimens from 22-day-old *Alpl*$^{-/-}$ mice treated with vehicle, 0.5 mg/kg/day ENB-0040 (Tx-0.5), or 2.0 mg/kg/day ENB-0040 (Tx-2.0), and untreated WT mice (radiographic magnification 5×). *Right panels:* μCT images of (A) femora and (B) tibiae of 22-day-old *Alpl*$^{-/-}$ mice treated with vehicle, Tx-0.5, or Tx-2.0 compared with untreated WT mice. Figures and legends taken from Yadav *et al*.[76] and reproduced with permission from the journal *Bone*.

notorious for their lack of vascularity. Such is the case of the enamel organ during tooth development. We have shown here that ENB-0040 reaches the enamel organ during the secretory and maturation phases in the treated *Alpl*$^{-/-}$ mice.[77] Scanning electron microscopy of *Alpl*$^{-/-}$ mice demonstrated a lack of organization of rod and inter-rod structure of enamel (Fig. 4). Micro-computed tomography showed that at 23 postnatal days, ENB-0040 has dose-dependent normalizing effects on both absolute and relative enamel volumes. Histological analysis of *Alpl*$^{-/-}$ mice showed reduced enamel mineralization both in molars and incisors, loss of polarization of ameloblasts that are important for enamel matrix formation, and even complete lack of enamel formation in the absence of TNAP. The fact that patients with odonto-HPP typically have the mildest reductions of serum TNAP levels[4] suggests that tooth formation is the most sensitive developmental process requiring TNAP function.[69, 74] Previous studies of cementum had demonstrated the sensitivity of this tissue to changes in the local P_i/PP_i ratio[78] and the ability of mineral-targeted TNAP to preserve acellular cementum in *Alpl*$^{-/-}$ mice had further validated that premise.[74] Here, too, the ability of ENB-0040 to preserve the structural integrity of enamel supports the notion that the enamel organ is under direct regulation by the extracellular P_i/PP_i ratio and that TNAP plays a crucial role in this regulation.[77]

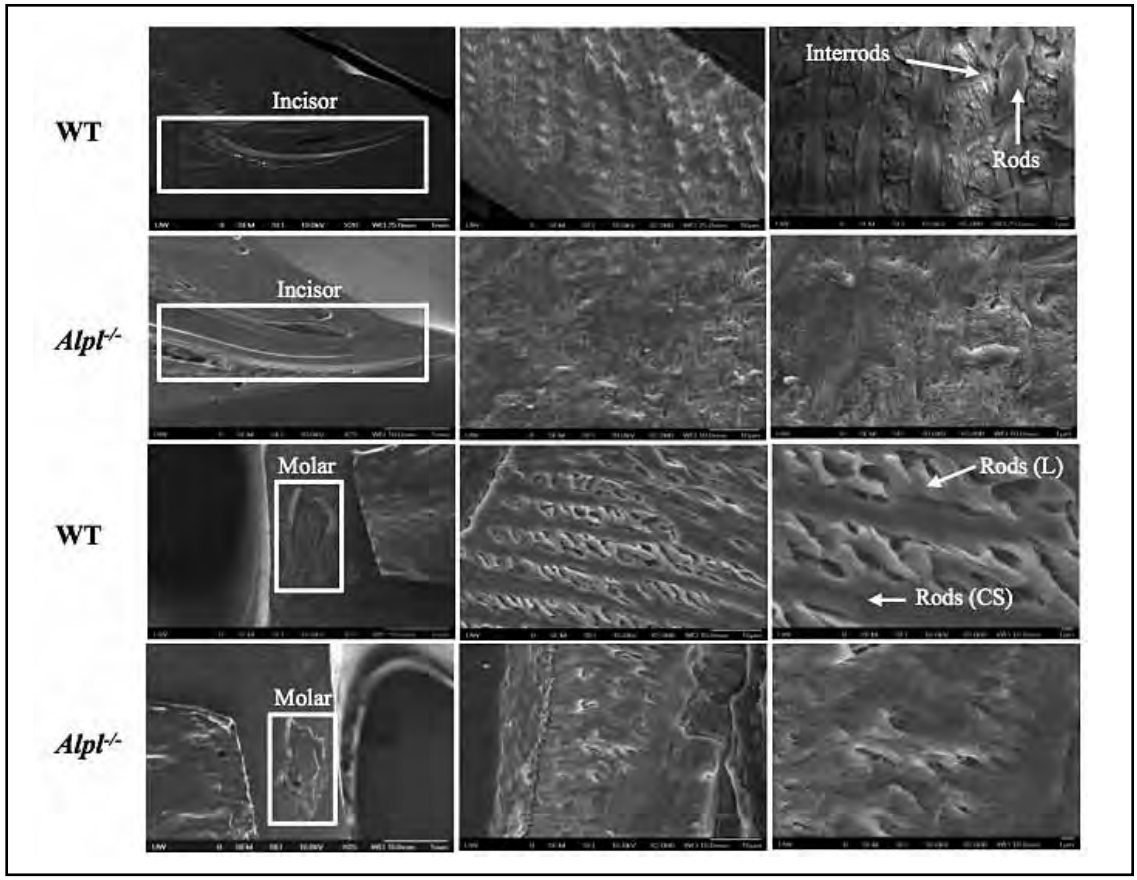

Figure 4. Scanning electron microscopy (SEM) analysis of incisors (top) and molars (bottom) of WT and *Alpl*-/- mice at 20 dpn. The SEM images showed well- decussated enamel rods and inter-rod in the molar crowns and crown analogs of incisors of WT mice. Note that there is a lack of rod–inter-rod organization in the *Alpl*$^{-/-}$ mice. The images were taken in the erupted part of the tooth. Figure taken from Yadav *et al.*[77] and reproduced with permission from the *Journal of Bone and Mineral Research.*

Preclinical validation of enzyme replacement for HPP using viral vector delivery of mineral-targeting TNAP

We have recently shown that a single injection of a lentiviral vector harboring TNAP-D_{10} (that is, soluble TNAP linked directly to D_{10}) resulted in sustained AP expression and phenotypic correction of HPP in *Alpl*$^{-/-}$ neonatal mice[79] including prevention of epileptic seizures and skeletal defects and preservation of a normal life-span. In another recent study, we demonstrated that a single intravenous injection of neonatal *Alpl*$^{-/-}$ mice with an adeno-associated viral vector expressing TNAP-D_{10} was as effective as the lentiviral vector treatment.[80] In both of these viral approaches, the Fc region was not included in the construct because neither purification of the enzyme nor an increase in the circulating plasma half-life was required. Indeed, these two viral delivery systems utilize either the liver (lentiviral vector) or skeletal muscle (adeno-associated viral vector) as factories of bone-targeted TNAP for the life of the animal. These delivery systems show promise as an alternative means of delivery of bone-targeted TNAP that would also decrease treatment frequency and potentially cost.

Furthermore, Matsumoto *et al.* demonstrated that TNAP lacking the D_{10} mineral-targeting domain could be an effective treatment for HPP mice, indicating that sustained and substantial TNAP production may be the most critical component of the treatment, more so than mineral-targeting.[80] More recently, Sugano and collaborators demonstrated the usefulness of viral vectors for delivery of mineral-targeting TNAP in utero, thus fetal therapy of HPP.[81] These very encouraging proof-of-principle preclinical studies could pave the way to gene therapy clinical trials using viral vectors as means of delivering mineral-targeting TNAP to patients. The potential rewards include the greatly reduced number of injection that a patient might need to receive in their lifetime and potentially reduced cost of the therapy. However, viral vector safety still remains a concern while EzRT is demonstrating a good track record of safety and efficacy.

Clinical trials using mineral-targeting TNAP for life-threatening HPP

An Investigational New Drug (IND) application to use asfotase alfa to treat HPP was filed in June of 2008. Initial dosing was then based on: 1) dose-response data of ENB-0040 in *Alpl*$^{-/-}$ mice,[76] 2) efficacy observed in treated *Alpl*$^{-/-}$ mice that achieved an equivalent to serum ALP activity in the range of 2,400-6,000 U/L; 3) the No-Observed-Adverse-Effect Level (NOAEL) in more sensitive species (rats and monkeys) established in one-month IV toxicology studies, and one-month IV/SC bridging and tolerability studies in rats; 4) a safety factor of 10 applied to the NOAEL; and 5) results from a multicenter, open-label, dose-escalating phase 1 study of the safety, tolerability, and pharmacology of ENB-0040 in six adults with HPP who received one dose of 3 mg/kg IV and then either 1 or 2 mg/kg ENB-0040 SC once weekly for three weeks. To date, three clinical studies have been completed, and three extension studies are ongoing, as well as an additional study in infants with HPP, currently recruiting patients (www.clinicaltrials.gov). The first full report of a clinical trial using asfotase alfa was on an open-label study to evaluate the safety, tolerability, bioavailability, pharmacokinetics, pharmacodynamics, and efficacy of treatment with asfotase alfa in severely affected infants and young children with HPP.[82] Efficacy assessments included radiographic skeletal changes, and changes in gross and fine motor function and cognitive development. The study included children 3 years old or less at enrollment, with signs of HPP occurring before

the age of 6 months, hypophosphatasemia, elevated plasma PLP levels, radiological signs of HPP, and failure to thrive. Other signs at baseline included rachitic chest deformity, pyridoxine-responsive seizures, history of a non-traumatic or poorly healing fractures, hypercalcemia, craniosynostosis, nephrocalcinosis, and respiratory compromise from HPP. The patients received asfotase alfa (at a concentration of 40 mg per milliliter) as a single intravenous infusion at a dose of 2 mg/kg, followed by subcutaneous injections three times per week at a dose of 1 mg/kg for 24 weeks, with an extension thereafter. The subcutaneous dose could be increased up to 3 mg/kg if there was worsening failure to thrive, deteriorating pulmonary function, or no radiographic evidence of skeletal improvement.[82]

Eleven patients were recruited.[82] One patient had a moderate reaction to the IV injection and the parents decided to discontinue the treatment. Another patient completed the first 6 months of treatment, but soon after succumbed to pneumonia and sepsis, judged unrelated to the study treatment. Of the nine patients who were treated for at least 1 year, four had perinatal HPP and five had infantile HPP. One had lost nearly all radiographically apparent skeletal mineral during prolonged ventilation and immobilization. Serum calcium levels were generally high at diagnosis (unlike calcium levels in other forms of rickets), and initially required dietary calcium restriction in all patients (most of whom had nephrocalcinosis). Skeletal remineralization occurred during treatment, including the patient mentioned above with extremely severe disease. Images of radiographic improvement are shown here for the oldest patient (3 years old) (Fig. 5) and for the youngest patient (<1 month old)

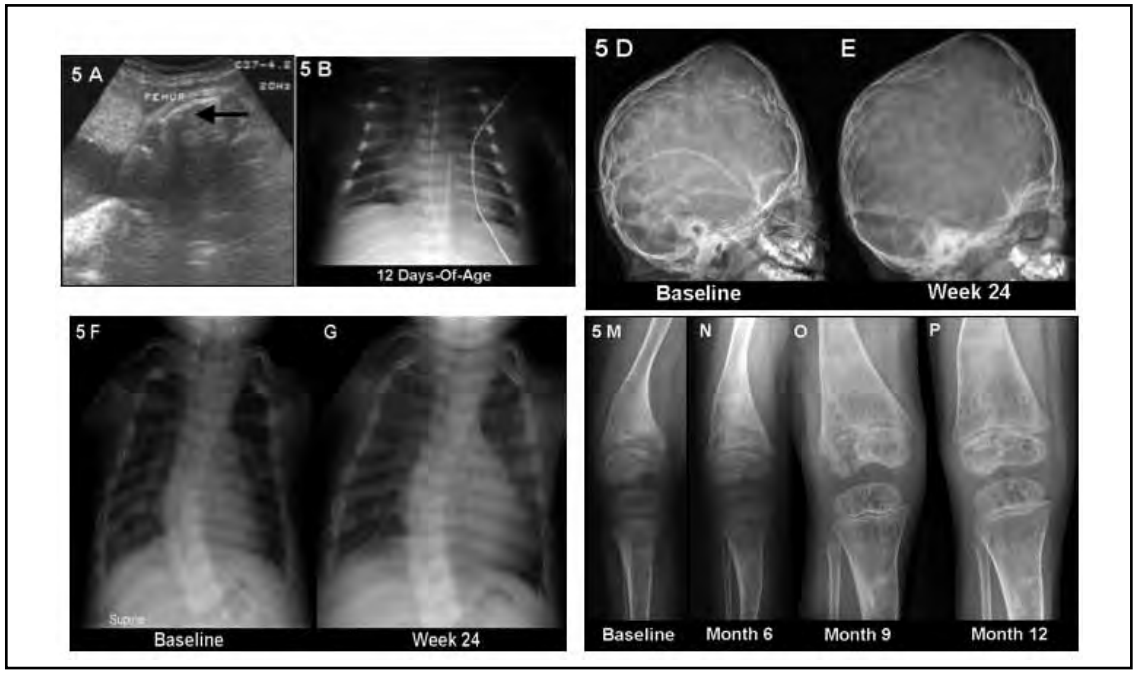

Figure 5. This 36-month-old girl had a short, bowed femur detected in utero by ultrasound (A). At 12 days-of-age, her chest radiograph showed thin, osteopenic ribs with lytic areas and fractures (B). Before treatment, severe pansuture closure was present, including a marked increase in "digital" markings ("beaten-copper" appearance) (D). The ribs at baseline were osteopenic and had fracture deformities with thin cortices (F). By week 24 of treatment, the ribs were wider and better mineralized with sharper cortical margins and less deformity (G). Improvement of the rickets with therapy is apparent (M, N, O, P). Images taken for the online supplementary data to Whyte *et al.*[82] and reproduced with permission from *The New England Journal of Medicine.*

(Fig. 6) at the start of the treatment. Treated infants showed no signs of symptomatic hypocalcemia from "hungry-bones" syndrome. Initially the treatment was often accompanied by increases in serum parathyroid hormone levels that called for liberalization of dietary calcium but not additional vitamin D.[82] A positive mineral balance throughout the skeleton was obvious on radiography after several weeks or months of treatment, and effects were evident in both membranous and endochondral bone. Substantial radiographic improvement in skeletal abnormalities was noted at week 24 in all but one patient, with continued healing through week 48. In the one patient who had no radiographically visible mineral at baseline, calcification was observed after 9 months of therapy. This delay probably reflected the profound deficit of skeletal mineral in the patient, although sufficient hydroxyapatite was apparently present for targeting with asfotase alfa. After the start of therapy, deciduous teeth erupted in all the patients, with only one patient having HPP-related loss of a tooth.[82]

In summary, of the 11 patients recruited, 10 completed 6 months of therapy; 9 completed 1 year. Healing of rickets was evident at 6 months in 9 patients. All patients showed improvement in developmental milestones

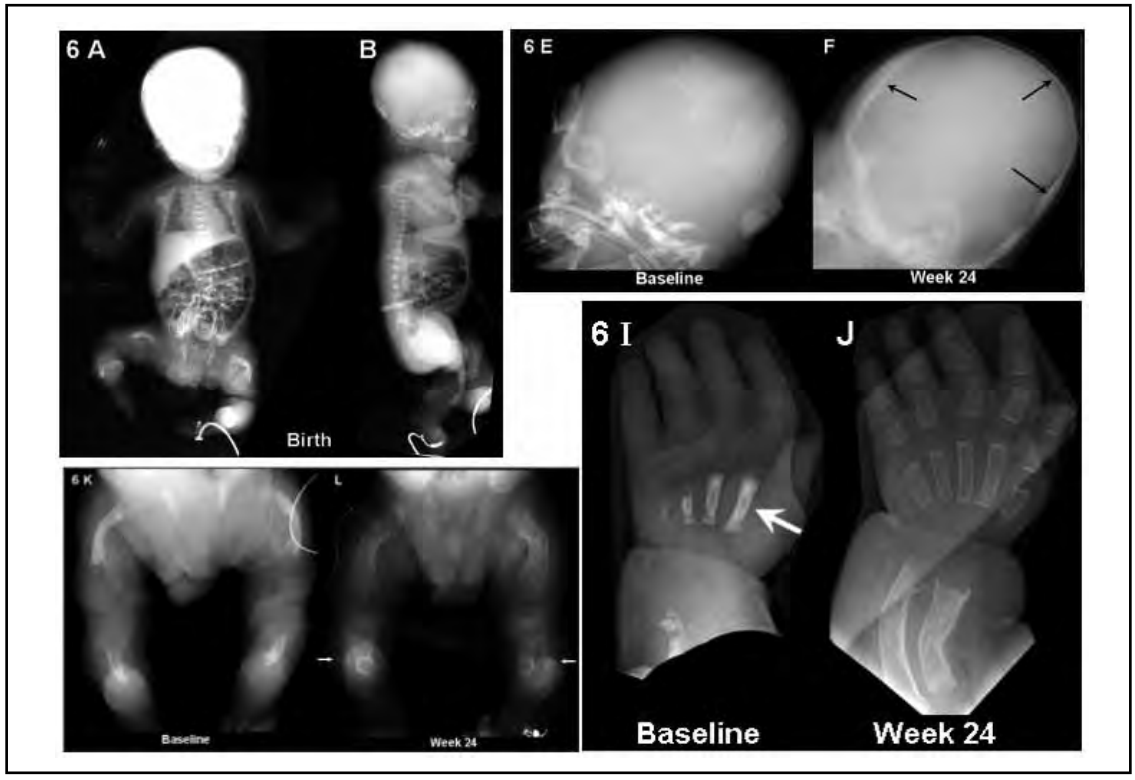

Figure 6. This 20-day-old (at therapy baseline) boy had shortened and bowed extremities and "fractures" detected by prenatal sonography at 17 – 18 weeks gestation (A, B). Before ENB-0040 treatment, little or decreased mineral was present in the frontal, parietal, or occipital bones, skull base, facial bones, and sphenoid (E). At week 24 of therapy, all areas showed striking remineralization (F). The improvement in the left hand and wrist was remarkable (I, J). Before ENB-0040, the femora were short, sclerotic, bowed, irregular, and lacked defined medullary cavities, cortices, and mineralized metaphyses and epiphyses. The fibulae were not calcified (K). After therapy, striking mineralization was evident (L). Images taken for the online supplementary data to Whyte *et al.*[82] and reproduced with permission from *The New England Journal of Medicine.*

and stabilization or improvement of pulmonary function. Elevated plasma levels of the TNAP substrates PP_i and PLP diminished. There was no evidence of symptomatic hypocalcemia, ectopic calcification, or definite drug-related serious adverse events. Low titers of anti–asfotase alfa antibodies developed in four patients, with no evident clinical, biochemical, or autoimmune abnormalities at 48 weeks of treatment. Thus, asfotase alfa therapy was associated with improved findings on skeletal radiographs and improved pulmonary and physical function in infants and young children with life-threatening HPP.

(Recibido: septiembre de 2012.
Aceptado: octubre 2012)

References

1. Fraser D. Hypophosphatasia. *Am J Med* 1957; 22:730-46.
2. Greenberg CR, Taylor CL, Haworth JC, Seargeant LE, Philipps S, Triggs-Raine B, Chodirker BN. A homoallelic Gly317-->Asp mutation in ALPL causes the perinatal (lethal) form of hypophosphatasia in Canadian mennonites. *Genomics* 1993; 17:215-7.
3. Millán JL. Mammalian alkaline phosphatases. From biology to applications in medicine and biotechnology. Weinheim, Germany: Wiley-VCH Verlag GmbH & Co; 2006.
4. Whyte M. Hypophosphatasia. In: Glorieux F, Jueppner H, Pettifor J, editors. Pediatric Bone. San Diego, CA: Elsevier (Academic Press); 2012. p. 771-94.
5. Fallon MD, Teitelbaum SL, Weinstein RS, Goldfischer S, Brown DM, Whyte MP. Hypophosphatasia: clinicopathologic comparison of the infantile, childhood, and adult forms. *Medicine (Baltimore)* 1984; 63:12-24.
6. Whyte MP, Teitelbaum SL, Murphy WA, Bergfeld MA, Avioli LV. Adult hypophosphatasia. Clinical, laboratory, and genetic investigation of a large kindred with review of the literature. *Medicine (Baltimore)* 1979; 58:329-47.
7. Whyte MP, Murphy WA, M. Fallon MD. Adult hypophosphatasia with chondrocalcinosis and arthropathy. Variable penetrance of hypophosphatasemia in a large Oklahoma kindred. *Am J Med* 1982; 72:631-41.
8. Wenkert D, Mcalister WH, Coburn SP, Zerega JA, Ryan LM, Ericson KL, Hersh JH, Mumm S, Whyte MP. Hypophosphatasia: nonlethal disease despite skeletal presentation in utero (17 new cases and literature review). *J Bone Miner Res* 2011; 26:2389-98.
9. Weiss MJ, Cole DE, Ray K, Whyte MP, Lafferty MA, Mulivor RA, Harris H. A missense mutation in the human liver/bone/kidney alkaline phosphatase gene causing a lethal form of hypophosphatasia. *Proc Natl Acad Sci USA* 1988; 85:7666-9.
10. Henthorn PS, Raducha M, Fedde KN, Lafferty MA, Whyte MP. Different missense mutations at the tissue-nonspecific alkaline phosphatase gene locus in autosomal recessively inherited forms of mild and severe hypophosphatasia. *Proc Natl Acad Sci USA* 1992; 89:9924-8.
11. Henthorn PS, Whyte MP. Missense mutations of the tissue-nonspecific alkaline phosphatase gene in hypophosphatasia. *Clin Chem* 1992; 38:2501-5.
12. Ozono K, Yamagata M, Michigami T, et al. Identification of novel missense mutations (Phe310Leu and Gly439Arg) in a neonatal case of hypophosphatasia. *J Clin Endocrinol Metab* 1996; 81:4458-61.

13. Orimo H, Hayashi Z, Watanabe A, Hirayama T, Hirayama T, Shimada T. Novel missense and frameshift mutations in the tissue-nonspecific alkaline phosphatase gene in a Japanese patient with hypophosphatasia. *Hum Mol Genet* 1994; 3:1683-4.
14. Mornet E, Taillandier A, Peyramaure S, et al. Identification of fifteen novel mutations in the tissue-nonspecific alkaline phosphatase (TNSALP) gene in European patients with severe hypophosphatasia. *Eur J Hum Genet* 1998; 6:308-14.
15. Mumm S, Jones J, Finnegan P, Whyte MP. Hypophosphatasia: molecular diagnosis of Rathbun's original case. *J Bone Miner Res* 2001; 16:1724-7.
16. Ali SY, Sajdera SW, Anderson HC. Isolation and characterization of calcifying matrix vesicles from epiphyseal cartilage. *Proc Natl Acad Sci USA* 1970; 67:1513-20.
17. Bernard GW. Ultrastructural localization of alkaline phosphatase in initial intramembranous osteogenesis. *Clin Orthop Relat Res* 1978; 218-25.
18. Morris DC, Masuhara K, Takaoka K, Ono K, Anderson HC. Immunolocalization of alkaline phosphatase in osteoblasts and matrix vesicles of human fetal bone. *Bone Miner* 1992; 19:287-98.
19. Anderson HC, Harmey D, Camacho NP, et al. Sustained osteomalacia of long bones despite major improvement in other hypophosphatasia-related mineral deficits in tissue nonspecific alkaline phosphatase/nucleotide pyrophosphatase phosphodiesterase 1 double-deficient mice. *Am J Pathol* 2005; 166:1711-20.
20. Anderson HC, Hsu HH, Morris DC, Fedde KN, Whyte MP. Matrix vesicles in osteomalacic hypophosphatasia bone contain apatite-like mineral crystals. *Am J Pathol* 1997; 151:1555-61.
21. Anderson HC, Sipe JB, Hessle L, et al. Impaired calcification around matrix vesicles of growth plate and bone in alkaline phosphatase-deficient mice. *Am J Pathol* 2004; 164:841-7.
22. Meyer JL. Can biological calcification occur in the presence of pyrophosphate? *Arch Biochem Biophys* 1984; 231:1-8.
23. Harmey D, Hessle L, Narisawa S, Johnson KA, Terkeltaub R, Millán JL. Concerted regulation of inorganic pyrophosphate and osteopontin by akp2, enpp1, and ank: an integrated model of the pathogenesis of mineralization disorders. *Am J Pathol* 2004; 164:1199-209.
24. Hessle L, Johnson KA, Anderson HC, et al. Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. *Proc Natl Acad Sci USA* 2002; 99: 9445-9.
25. Murshed M, Harmey D, Millán JL, Mckee MD, Karsenty G. Unique coexpression in osteoblasts of broadly expressed genes accounts for the spatial restriction of ECM mineralization to bone. *Genes Dev* 2005; 19:1093-104.
26. Johnson KA, Hessle L, Vaingankar S, et al. Osteoblast tissue-nonspecific alkaline phosphatase antagonizes and regulates PC-1. *Am J Physiol Regul Integr Comp Physiol* 2000; 279:R1365-77.
27. Ciancaglini P, Yadav MC, Simao AM, et al. Kinetic analysis of substrate utilization by native and TNAP-, NPP1-, or PHOSPHO1-deficient matrix vesicles. *J Bone Miner Res* 2010; 25:716-23.
28. Majeska RJ, Wuthier RE. Studies on matrix vesicles isolated from chick epiphyseal cartilage. Association of pyrophosphatase and ATPase activities with alkaline phosphatase. *Biochim Biophys Acta* 1975; 391:51-60.
29. Moss DW, Eaton RH, Smith JK, Whitby LG. Association of inorganic-pyrophosphatase activity with human alkaline-phosphatase preparations. *Biochem J* 1967; 102:53-7.
30. Rezende AA, Pizauro JM, Ciancaglini P, Leone FA. Phosphodiesterase activity is a novel property of alkaline phosphatase from osseous plate. *Biochem J* 1994; 301 (Pt 2):517-22.
31. Robison R. The Possible Significance of Hexosephosphoric Esters in Ossification. *Biochem J* 1923; 17:286-93.
32. Yadav MC, Simao AM, Narisawa S, et al. Loss of skeletal mineralization by the simultaneous ablation of PHOSPHO1 and alkaline phosphatase function: a unified model of the mecha-

nisms of initiation of skeletal calcification. *J Bone Miner Res* 2011; 26:286-97.
33. Coburn SP. Modeling vitamin B6 metabolism. *Adv Food Nutr Res* 1996; 40:107-32.
34. Jansonius JN. Structure, evolution and action of vitamin B6-dependent enzymes. *Curr Opin Struct Biol* 1998; 8:759-69.
35. Whyte MP, Landt M, Ryan LM, et al. Alkaline phosphatase: placental and tissue-nonspecific isoenzymes hydrolyze phosphoethanolamine, inorganic pyrophosphate, and pyridoxal 5'-phosphate. Substrate accumulation in carriers of hypophosphatasia corrects during pregnancy. *J Clin Invest* 1995; 95:1440-5.
36. Chodirker BN, Coburn SP, Seargeant LE, Whyte MP, Greenberg CR. Increased plasma pyridoxal-5'-phosphate levels before and after pyridoxine loading in carriers of perinatal/infantile hypophosphatasia. *J Inherit Metab Dis* 1990; 13:891-6.
37. Whyte MP, Mahuren JD, Fedde KN, Cole FS, Mccabe ER, Coburn SP. Perinatal hypophosphatasia: tissue levels of vitamin B6 are unremarkable despite markedly increased circulating concentrations of pyridoxal-5'-phosphate. Evidence for an ectoenzyme role for tissue-nonspecific alkaline phosphatase. *J Clin Invest* 1988; 81:1234-9.
38. Whyte MP, Mahuren JD, Vrabel LA, Coburn SP. Markedly increased circulating pyridoxal-5'-phosphate levels in hypophosphatasia. Alkaline phosphatase acts in vitamin B6 metabolism. *J Clin Invest* 1985; 76:752-6.
39. Narisawa S, Frohlander N, Millán JL. Inactivation of two mouse alkaline phosphatase genes and establishment of a model of infantile hypophosphatasia. *Dev Dyn* 1997; 208:432-46.
40. Narisawa S, Wennberg C, Millán JL. Abnormal vitamin B6 metabolism in alkaline phosphatase knock-out mice causes multiple abnormalities, but not the impaired bone mineralization. *J Pathol* 2001; 193:125-33.
41. Waymire KG, Mahuren JD, Jaje JM, Guilarte TR, Coburn SP, Macgregor GR. Mice lacking tissue non-specific alkaline phosphatase die from seizures due to defective metabolism of vitamin B-6. *Nat Genet* 1995; 11:45-51.
42. Fleshood HL, Pitot HC. O-phosphorylethanolamine ammonia lyase, a new pyridoxal phosphate-dependent enzyme. *Biochem Biophys Res Commun* 1969; 36:110-8.
43. Fleshood HL, Pitot HC. The metabolism of O-phosphorylethanolamine in animal tissues. II. Metabolic regulation of O-phosphorylethanolamine phospho-lyase *in vivo*. *Arch Biochem Biophys* 1970; 141:423-9.
44. Millán JL, Whyte MP, Avioli LV, Fishman WH. Hypophosphatasia (adult form): quantitation of serum alkaline phosphatase isoenzyme activity in a large kindred. *Clin Chem* 1980; 26:840-5.
45. Harmey D, Johnson KA, Zelken J, et al. Elevated skeletal osteopontin levels contribute to the hypophosphatasia phenotype in Akp2(-/-) mice. *J Bone Miner Res* 2006; 21:1377-86.
46. Johnson K, Goding J, Van Etten D, et al. Linked deficiencies in extracellular PP(i) and osteopontin mediate pathologic calcification associated with defective PC-1 and ANK expression. *J Bone Miner Res* 2003; 18: 994-1004.
47. Wennberg C, Hessle L, Lundberg P, et al. Functional characterization of osteoblasts and osteoclasts from alkaline phosphatase knockout mice. *J Bone Miner Res* 2000; 15:1879-88.
48. Goldberg HA, Warner KJ, Li MC, Hunter GK. Binding of bone sialoprotein, osteopontin and synthetic polypeptides to hydroxyapatite. *Connect Tissue Res* 2001; 42:25-37.
49. Christensen B, Nielsen MS, Haselmann KF, Petersen TE, Sorensen ES. Post-translationally modified residues of native human osteopontin are located in clusters: identification of 36 phosphorylation and five O-glycosylation sites and their biological implications. *Biochem J* 2005; 390:285-92.
50. Hunter GK, Kyle CL, Goldberg HA. Modulation of crystal formation by bone phosphoproteins: structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation. *Biochem J* 1994; 300 (Pt 3):723-8.
51. Jono S, Peinado C, Giachelli CM. Phosphorylation of osteopontin is required for inhibition of vascular smooth muscle cell calcification. *J Biol Chem* 2000; 275:20197-203.

52. Pampena DA, Robertson KA, Litvinova O, Lajoie G, Goldberg HA, Hunter GK. Inhibition of hydroxyapatite formation by osteopontin phosphopeptides. *Biochem J* 2004; 378:1083-7.
53. Addison WN, Masica DL, Gray JJ, Mckee MD. Phosphorylation-dependent inhibition of mineralization by osteopontin ASARM peptides is regulated by PHEX cleavage. *J Bone Miner Res* 2010; 25:695-705.
54. Deeb AA, Bruce SN, Morris AA, Cheetham TD. Infantile hypophosphatasia: disappointing results of treatment. *Acta Paediatr* 2000; 89:730-3.
55. Sutton RA, Mumm S, Coburn SP, Ericson KL, Whyte MP. "Atypical femoral fractures" during bisphosphonate exposure in adult hypophosphatasia. *J Bone Miner Res* 2012; 27:987-94.
56. Cahill RA, Wenkert D, Perlman SA, et al. Infantile hypophosphatasia: transplantation therapy trial using bone fragments and cultured osteoblasts. *J Clin Endocrinol Metab* 2007; 92:2923-30.
57. Whyte MP, Kurtzberg J, Mcalister WH, et al. Marrow cell transplantation for infantile hypophosphatasia. *J Bone Miner Res* 2003; 18:624-36.
58. Camacho PM, Painter S, Kadanoff R. Treatment of adult hypophosphatasia with teriparatide. *Endocr Pract* 2008; 14:204-8.
59. Doshi KB, Hamrahian AH, Licata AA. Teriparatide treatment in adult hypophosphatasia in a patient exposed to bisphosphonate: a case report. *Clin Cases Miner Bone Metab* 2009; 6:266-9.
60. Schalin-Jantti C, Mornet E, Lamminen A, Valimaki MJ. Parathyroid hormone treatment improves pain and fracture healing in adult hypophosphatasia. *J Clin Endocrinol Metab* 2010; 95: 5174-9.
61. Whyte MP, Mumm S, Deal C. Adult hypophosphatasia treated with teriparatide. *J Clin Endocrinol Metab* 2007; 92:1203-8.
62. Gagnon C, Sims NA, Mumm S, et al. Lack of sustained response to teriparatide in a patient with adult hypophosphatasia. *J Clin Endocrinol Metab* 2010; 95:1007-12.
63. Laroche M. Failure of teriparatide in treatment of bone complications of adult hypophosphatasia. *Calcif Tissue Int* 2012; 90:250.
64. Vahle JL, Long GG, Sandusky G, Westmore M, Ma YL, Sato M. Bone neoplasms in F344 rats given teriparatide [rhPTH(1-34)] are dependent on duration of treatment and dose. *Toxicol Pathol* 2004; 32:426-38.
65. Chung TD, Sergienko E, Millán JL. Assay format as a critical success factor for identification of novel inhibitor chemotypes of tissue-nonspecific alkaline phosphatase from high-throughput screening. *Molecules* 2010; 15:3010-37.
66. Whyte MP, Valdes Jr. R, Ryan LM, McAlister WH. Infantile hypophosphatasia: enzyme replacement therapy by intravenous infusion of alkaline phosphatase-rich plasma from patients with Paget's bone disease. *J Pediatr* 1982; 101:379-386.
67. Whyte MP, Mcalister WH, Patton LS, et al. Enzyme replacement therapy for infantile hypophosphatasia attempted by intravenous infusions of alkaline phosphatase-rich Paget plasma: results in three additional patients. *J Pediatr* 1984; 105:926-33.
68. Weninger M, Stinson RA, Plenk H, Jr., Bock P, Pollak A. Biochemical and morphological effects of human hepatic alkaline phosphatase in a neonate with hypophosphatasia. *Acta Paediatr Scand* 1989; 360(Suppl):154-60.
69. Millán JL, Narisawa S, Lemire I, et al. Enzyme replacement therapy for murine hypophosphatasia. *J Bone Miner Res* 2008; 23:777-87.
70. Kasugai S, Fujisawa R, Waki Y, Miyamoto K, Ohya K. Selective drug delivery system to bone: small peptide (Asp)6 conjugation. *J Bone Miner Res* 2000; 15:936-43.
71. Nishioka T, Tomatsu S, Gutierrez MA, et al. Enhancement of drug delivery to bone: characterization of human tissue-nonspecific alkaline phosphatase tagged with an acidic oligopeptide. *Mol Genet Metab* 2006; 88:244-55.
72. Yokogawa K, Miya K, Sekido T, et al. Selective delivery of estradiol to bone by aspartic acid oligopeptide and its effects on ovariectomized mice. *Endocrinology* 2001; 142:1228-33.

73. Fedde KN, Blair L, Silverstein J, et al. Alkaline phosphatase knock-out mice recapitulate the metabolic and skeletal defects of infantile hypophosphatasia. *J Bone Miner Res* 1999; 14:2015-26.
74. Mckee MD, Nakano Y, Masica DL, et al. Enzyme replacement therapy prevents dental defects in a model of hypophosphatasia. *J Dent Res* 2011; 90:470-6.
75. Foster BL, Nagatomo KJ, Tso HW, et al. Tooth root dentin mineralization defects in a mouse model of hypophosphatasia. *J Bone Miner Res* 2012; In press.
76. Yadav MC, Lemire I, Leonard P, et al. Dose response of bone-targeted enzyme replacement for murine hypophosphatasia. *Bone* 2011; 49:250-6.
77. Yadav MC, De Oliveira RC, Foster BL, et al. Enzyme replacement prevents enamel defects in hypophosphatasia mice. *J Bone Miner Res* 2012; 27:1722-34.
78. Nociti FH, Jr., Berry JE, Foster BL, Get al. Cementum: a phosphate-sensitive tissue. *J Dent Res* 2002; 81:817-21.
79. Yamamoto S, Orimo H, Matsumoto T, et al. Prolonged survival and phenotypic correction of Akp2(-/-) hypophosphatasia mice by lentiviral gene therapy. *J Bone Miner Res* 2011; 26:135-42.
80. Matsumoto T, Miyake K, Yamamoto S, et al. Rescue of severe infantile hypophosphatasia mice by AAV-mediated sustained expression of soluble alkaline phosphatase. *Hum Gene Ther* 2011; 22:1355-64.
81. Sugano H, Matsumoto T, Miyake K, et al. Successful gene therapy in utero for lethal murine hypophosphatasia. *Hum Gene Ther* 2012; 23:399-406.
82. Whyte MP, Greenberg CR, Salman NJ, et al. Enzyme-replacement therapy in life-threatening hypophosphatasia. *N Engl J Med* 2012; 366:904-13.