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Abstracts from the Second Scientific Meeting of Soft Bones Inc., June 2-4, 2023, Bethesda, MD, USA

Soft Bones
Finding the Key to HPP

2023 SCIENTIFIC MEETING

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SECOND SCIENTIFIC MEETING

The Second Scientific Meeting of Soft Bones Inc., held June 2 - 4, 2023, in Bethesda, MD, USA, brought together clinicians, physician-scientists, basic science researchers, and industry representatives from across the globe to better understand and to improve the treatment of hypophosphatasia (HPP). Attendees shared the latest advances and discussed ongoing and future research concerning this rare and complex inborn-error-of-metabolism that manifests the broadest range of severity of all skeletal diseases. The Scientific Planning Committee members included Michael P. Whyte, MD, Committee Chairman, Washington University School of Medicine, Kathryn Dahir, MD, Vanderbilt University Medical Center, Cheryl Rockman-Greenberg, MD, Max Rady College of Medicine University of Manitoba, José Luis Millán, PhD, Sanford Children's Health Research Center, and Peter Tebben, MD, Mayo Clinic.

The meeting began by delving into the etiology of HPP, therefore focusing on its genetics. Wolfgang Högler, MD updated the importance of the ALPL gene variant database, and Carlos Ferreira, MD discussed the genetics underlying inorganic pyrophosphate (PPi) synthesis and metabolism. Deborah Wenkert, MD talked about the HPP phenome, including environmental factors that can impact the HPP phenotype. Richard Eastell, MD and Nilton Salles Rosa Neto, MD, PhD then shared insights for identifying and distinguishing in the clinical laboratory "carriers" versus those with HPP.

Advances in understanding the complications of HPP began with Marc McKee, PhD who explored fundamental aspects of skeletal mineralization. Patrick Mantyh, PhD discussed how we can understand the pathophysiology of skeletal pain, a common HPP symptom in adults as emphasized by Chad Deal, MD. How to distinguish HPP from osteoporosis was highlighted by Peter Tebben, MD. Next, Gary Gottesman, MD and Eric Rush, MD emphasized uncertainties when first encountering patients with skeletal diseases that resemble HPP. The significant contributions of orthopedists to the diagnosis and treatment of HPP, in both children and adults, were highlighted by Laura Tosi, MD and Lothar Seefried, MD, respectively.

Subsequently, Brian Foster, PhD described important new mouse models for the dental phenotype of HPP. Mark Rallo, OD assessed the deposits of calcium that can occur on the surface of eyes from HPP. New research in HPP illustrated how far the understanding of HPP pathogenesis has come in recent years with Dana Gaddy, PhD and Victoria DeMambro, PhD Candidate, discussing emerging evidence of disturbed energy metabolism in muscle and adipose tissue, respectively.

Aspects of HPP treatment were presented by Cheryl Rockman-Greenberg, MD and Kathryn Dahir, MD who reinforced the importance of maintaining enzyme replacement (asfotase alfa) therapy, and Donna Griffin, DPT outlined how to use functional testing to assess responses to treatment. Tae Matsumoto, MD, PhD shared details concerning mouse models of HPP, and treating severe HPP prenatally. Michael Whyte, MD detailed that placental alkaline phosphatase can hydrolyze the HPP natural substrates in pregnant women. José Luis Millán, PhD described prospects for developing a gene therapy for HPP.

The recipients of Soft Bones Foundation research grants reviewed what this support helped them achieve: Steve Mumm, PhD, Washington University School of Medicine (2014), Luke Mortensen PhD, The University of Georgia (2015), Brian Foster, PhD, The Ohio State University College of Dentistry (2016, 2018), Kathryn Dahir, MD, Vanderbilt University Medical Center - (recorded presentation by Co-Pi Michael de Riesthal, PhD, CCC-SLP) (2019), Dobrawa Napierala, PhD, University of Pittsburgh School of Dental Medicine (2020), Dana Gaddy, PhD, Texas A&M University (2020), Flavia Amadeu de Oliveira, PhD, Sanford Burnham Prebys Medical Discovery Institute (2021), Fatma F. Mohamed, PhD, The Ohio State University College of Dentistry (2022), and Nan Hatch, DMD, PhD, University of Michigan School of Dentistry (2023).

To conclude, representatives from pharmaceutical or biotech companies reviewed emerging considerations for advancing the treatment of HPP: Derek Dunn, M.Med.Sci., Alexion AstraZeneca; Lothar Seefried, MD, University Wuerzburg, Germany (AM-Pharma); Rick Morgan, PhD, BeBio; Tae Matsumoto, MD, PhD, Nippon Medical School (Aruvant Sciences); Prof. Takeshi Taketani, MD, PhD, Shimane University (PuREC); and Jeffrey Bartlett, PhD, Rampart Bio.

We thank the session moderators Nick Bishop, MD, University of Sheffield, Susan Ott, MD, University of Washington, Tom Weber, MD, Duke University Medical Center, Larry Suva, PhD, Texas A&M University, and Alison Boyce, MD, NIH – NIDCR,

This Second Scientific meeting was organized by Deborah Fowler, President and Founder of the Soft Bones Foundation, and assisted by Denise Goodbar, LeighAnne Castimore, Katie Kelly, Sue Krug, Ellen Reppe, Cindy Reasor, and consultant, Charlene Waldman, along with an on-site technical team. Special thanks to the sponsors: Alexion AstraZeneca Rare Disease, AM Pharma, Charles River, PuREC, Rallybio, RampartBio Be Biopharma, and 1cBio.

Deborah Fowler
Founder and President

Michael P. Whyte, MD
Chair, Scientific Advisory Board

Soft Bones Foundation
Boonton, NJ, USA https://www.softbones.org/

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ALPL Gene Variant Database: Current Construction, Validation, and Interpretation

Wolfgang Högler, MD, FRCPCH

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Hypophosphatasia (HPP) is an inherited multisystem disorder predominantly affecting the mineralization of bones and teeth. HPP is caused by pathogenic variants in ALPL, which encodes tissue non-specific alkaline phosphatase (TNSALP). The ALPL gene variant database (https://alplmutationdatabase.jku.at/) is an open-access archive for interpretations of the clinical significance of variants reported in ALPL.

The database contains coding and non-coding variants, including single nucleotide variants, insertions/deletions and structural variants affecting coding or intronic sequences of ALPL. Each variant in the database is displayed with details explaining the corresponding pathogenicity, and all reported genotypes and phenotypes, including references. In 2021, the ALPL gene variant classification project was established to reclassify variants of uncertain significance (VUS) and continuously assess and update genetic, phenotypic, and functional variant information in the database.

For this purpose, the database provides a unique submission system for clinicians, geneticists, genetic counsellors and researchers to submit VUS within ALPL for classification. An international, multidisciplinary consortium of HPP experts has been established to reclassify the submitted VUS using a multi-step process adhering to the stringent ACMG/AMP variant classification guidelines.

These steps include a clinical phenotype assessment, deep literature research including artificial intelligence technology, molecular genetic assessment, and in-vitro functional testing of variants in a co-transfection model to measure ALP residual activity. This classification project and the ALPL gene variant database will serve the global medical community, widen the genotypic and phenotypic HPP spectrum by reporting and characterizing new ALPL variants based on ACMG/AMP criteria and thus facilitate improved genetic counselling and medical decision making for affected patients and families. The project may also serve as a gold standard framework for multidisciplinary collaboration for variant interpretation in other rare diseases.

Clinical, Biochemical, And Genetic Studies Of Pediatric Hypophosphatasia "Carrier" Parents

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Gary S. Gottesman1, William H. McAlister3, Karen L. Ericson4, Stephen P. Coburn4, Michael P. Whyte1,2.

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In hypophosphatasia (HPP), deficient tissue non-specific alkaline phosphatase (TNSALP) causes endogenous accumulation of its natural substrates, including pyridoxal 5´-phosphate (PLP). Diagnosing HPP requires clinical acumen recognizing the broad-ranging onset and severity of its signs, symptoms, and complications. Especially challenging for "carriers" of specific *ALPL* pathogenic variants is whether they might develop overt disease. Using clinical, biochemical, radiological, and molecular analyses, we characterized >250 children with HPP [174 families] and investigated many of their relatives. Herein, we interpret the parental levels of serum ALP, plasma PLP, and their *ALPL* findings. There were 169 mothers and 133 fathers; mean age 34(±7) [range: 20-55 years] and 36(±7) [range: 22-63 years], respectively. Among them, 130 (43%) harbored at least one *ALPL* pathogenic variant. In thirty-five parents (12%) no pathogenic *ALPL* variant was found. For the remaining 137 parents, despite availability of biochemical results, *ALPL* analysis is incomplete. Fifty-five parents (18.2%) were considered "affected" by HPP, i.e., manifested clinical features as well as an *ALPL* defect, or combination of laboratory evidence and autosomal dominant inheritance. In contrast, 141 parents (46.7%) were "unaffected carriers", i.e., no clinical features of HPP, but, both low ALP and elevated PLP or, if studied, an *ALPL* pathogenic variant. Finally, 106 parents (35.1%) were considered "controls", i.e., neither an obligate carrier nor genetic or serologic evidence of HPP. Mean(±SD) ALP [reference range 40.6–110.4 U/L] was 30(±9) in "affected", 36(±12) in "unaffected carrier", and 65(±24) in "control" parents, $p<0.001$. Mean(±SD) PLP [reference range 5-107 nM] was 306(±180) in "affected", 192(±155) in "unaffected carrier", and 78(±61) in "control" parents, $p<0.001$. Thus, among these 302 parents, 196 manifested biochemical findings of HPP and/or a pathogenic variant in *ALPL*, but only 55 (18.2%) had clinical evidence of overt disease. This clinical experience seems useful for identifying *ALPL* defects that harbor a dominant negative effect.

Pyrophosphate: Homeostasis and Aberrations

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Pyrophosphate consists of two orthophosphate anions in a P-O-P linkage. It was discovered in 1827, as its name implies, by heating sodium salts of orthophosphate. Its formation in a biological system was first identified in 1941 by Cori. Here, we review the dozens of enzymatic reactions that produce pyrophosphate, and the few that degrade it, both inside and outside the cell. We review the role of pyrophosphate in the synthesis of biological macromolecules, in mineralization, and in the post-translational modification of proteins. We also briefly discuss the few disorders associated with aberrant pyrophosphate homeostasis, leading to decreased extracellular concentration (ENPP1 deficiency and ABCC6 deficiency), excess (hypophosphatasia), abnormal intracellular metabolism (inorganic pyrophosphatase 2 deficiency), and dysregulated transport (deficiency of ANKH inorganic pyrophosphate transport regulator).

The Phenome and HPP

Deborah Wenkert, MD

Wenkert & Young, LLC Thousand Oaks, CA

Dominant vs. recessive inheritance, and the specific *ALPL* variant(s) explain much of a HPP phenotype. In vitro, mouse, and human studies offer insight regarding the interactions of genes, epigenetics, symbiotic microorganisms, environmental exposures (the phenome) on the skeleton, and may explain differences seen between patients carrying the same ALPL mutation(s).

Gene-Gene:

- Mouse Alpl-/- crossed with Enpp1-/- or Ank-/- ameliorates skeletal disease
- Child with HPP plus mutation of
  - FGFR3 disproportionate short stature (hypochondroplasia) and Bowdler spurs (HPP)
  - COL1A1 postnatal fractures as a toddler (OI) and features of OI and osteomalacia (HPP) on bone biopsy

Epigenome (modifiers of DNA expression: DNA methylation, histone modifications…):

- In vitro inhibition of histone acetylates (I and II) in articular chondrocytes downregulates ENPP1 and ANKH and upregulates TNAP gene expression

Microbiome:

- The elevated bone mass of germfree mice normalizes with normal gut microbiota colonization

Environment:

- Fetal crowding in HPP and postnatal excess weight may exacerbate long bone bowing.
- Significant [PPi] increases occur after intense exercise in healthy volunteers
- ALP activity suppression can occur in celiac disease, hypothyroidism, or profound anemia

Diet and medication:

- Other forms of osteomalacia occur from dietary insufficiency of calcium, phosphate, or vitamin D
- ALP activity can be suppressed in otherwise healthy individuals with Clofibrate, excess glucocorticoids or vitamin D, deficiency of ALP cofactors (Zn, Mg), deficiency of vitamin C, or starvation
- Phosphorus, a competitive inhibitor of ALP, is high-normal or elevated in HPP from elevated urinary reclamation (with low FGF7 and normal FGF23 and sFRP4 levels). Measurement of ALP substrates in children with HPP given 3-days of phosphate supplementation (~20-60 mg/kg; n=7) or dietary restriction (1/2 RDA; n=7) were consistent with phosphorus inhibition of ALP in HPP. Those supplemented with phosphorus showed a 56% increase from baseline in urinary PPi levels (nominal p=0.03), a trend toward elevated plasma PLP levels, and a significant difference in urinary PPi levels compared to the phosphorus restricted cohort (nominal p=0.0036).

The *Stenciling Principle* for Mineralization: What is it and what is its Importance?

Marc D. McKee, PhD and Natalie Reznikov, PhD

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The *Stenciling Principle* for extracellular matrix mineralization acts across many levels, from the macroscale (skeleton/dentition vs soft connective tissues), to the mesoscale (*e.g*. entheses, periodontal ligament), and to the microscale (mineral tessellation). Based on Jacob and Monod's 19611 pivotal paradigm "repressing a repressor" to induce an activation effect – as originally explaining genetic regulation of enzyme expression in bacteria – the stenciling principle describes an enzyme/substrate-based, double-negative regulatory process ("inhibition of inhibitors") that promotes mineralization in bone and other mineralized tissues (in contrast to the default condition of inhibition alone that prevents mineralization in soft connective tissues). The stenciling principle relates to both wide-ranging, pervasive small-molecule coarse inhibition of mineralization (*e.g*. by pyrophosphate), and to finer and sustained protein inhibition/regulation (*e.g*. by osteopontin) to refine and guide mineralization, with both involving promoters (enzymes, *e.g.* TNAP, PHEX) that inhibit/remove-the-inhibition to permit and regulate mineralization. In this process, an organizational microscale motif for bone mineral arises called *crossfibrillar mineral tessellation* where microscale mineral formations called *tesselles* (geometrically approximating prolate ellipsoids) traverse multiple collagen fibrils laterally. Tesselle growth is directed by the structural anisotropy of collagen, being spatially restrained in the shorter transverse tesselle dimension (tesselles average 1.6 x 0.8 x 0.8 µm, aspect ratio 2, length range 1.5-2.5 µm). Temporo-spatially, the tesselles abut in 3D (close ellipsoid packing) to fill the volume of bone extracellular matrix. Discrete interfacial gaps between adjacent tesselles remain discernable even in mature lamellar bone. Volume-filling of extracellular matrix with a repeating mineral structural unit results in numerous patterned interfaces distributed in three dimensions which allows for dissipation of critical stresses, and enables fail-safe cyclic loading through minute collective deformations at these interfaces. Incomplete or defective mineralization or mineral tessellation patterning in osteomalacic bone indubitably leads to bone deformities with bowing, buckling and/or fracture along with compromised mechanical performance under loading. 1J.Mol.Biol.3(3),318-356.

Pain: Is it in Your Muscles and/or Bones? What Explains Bone Pain?

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Injury / disorders of the skeleton in the young, adult, and aged humans are frequently accompanied by chronic pain. Considerable progress has been made in understanding the unique population of sensory and sympathetic nerves that innervate bone, muscle, cartilage, and tendons. Sensory nerve fibers were previously viewed as simple static structures that detected injury.

However, recent data has shown that sensory fibers that innervate the skeleton are remarkably adaptive. Thus, both sensory neurons and Central Nervous System brain pathways change their morphology, phenotype, and sensitivity to amplify pain following skeletal injury. Results from animal models have frequently predicted results obtained in human clinical trials and that a therapy that relieves one type of skeletal pain frequently attenuates other types of skeletal pain. Translating these advance into approved new therapies may change how we effectively manage skeletal pain in young, adult, and aging patients.

The Challenges of Diagnosing HPP: Molecular Diagnosis is Never Enough

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Diagnosis of hypophosphatasia (HPP) is often nuanced and relies on the clinical acumen of the clinician. HPP has an extraordinary range of variability in phenotypic expression, spanning extreme hypomineralization with dysfunctional skeletal mechanics leading to fetal death to adult onset joint disease or dental problems without apparent skeletal abnormality. This extensive phenotypic variability can occur within families among members who harbor identical *ALPL* mutation(s). Autosomal dominant and autosomal recessive inheritance patterns are documented. Even so, molecular support for the diagnosis may be challenging when rare alterations in *ALPL* occur outside the coding regions and exon/intron boundaries analyzed in commercial laboratories. Further complicating the clinician's efforts are the various other causes of hypophosphat***asemia*** (e.g., cleidocranial dysplasia, hypothyroidism, zinc or magnesium deficiency, celiac disease, etc.).

Recent insightful biochemical, radiologic and histologic evaluations from our clinics at Washington University School of Medicine and the St. Louis Children's Hospital explore the diagnostic challenges HPP offers. Examples include: 1) Maternal vitamin B6 deficiency masking confirmatory biochemistries of perinatal HPP; 2) Affected patients whose parents and siblings have biochemical evidence of abnormalities but have no overt disease; 3) Chronic glucocorticoid exposure for Duchenne muscular dystrophy associated with hypophosphatasemia requiring *ALPL* mutation analysis before starting anti-resorptive therapy; 4) A child with a single *ALPL* variant without overt skeletal findings, whose low ALP activity led to the surprising molecular finding of two *ALPL* variants in her apparently unaffected mother.

Hypophosphatasia or Osteoporosis: A potential clinical conundrum

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Osteoporosis is common amongst postmenopausal women and older men and is characterized by low bone density and increased risk of fracture. Hypophosphatasia (HPP) is a rare, heritable condition caused by loss of function variants in the gene encoding tissue nonspecific alkaline phosphatase and leads to abnormal bone mineralization. The spectrum of severity is broad ranging from early deciduous tooth loss to life-threatening disease in the perinatal and infantile forms. Although the underlying pathophysiology of HPP is distinct from that of osteoporosis, clinical overlap exists between these conditions including the occurrence of low trauma fractures and low bone mass. These common features create the opportunity for misdiagnosis and inappropriate therapeutic intervention. Several reports describe patients with unrecognized HPP treated with antiresorptive medication for presumed osteoporosis resulting in atypical fractures. As such, antiresorptive treatment is contraindicated in HPP.

Adults with HPP are comprised of two main groups, 1) those with childhood-onset symptoms and 2) those with symptom onset during adulthood. Common features of adult-onset disease include musculoskeletal pain, fractures/pseudofractures of the lower extremities, and dental abnormalities. Because HPP is rare, clinical awareness is often insufficient and low alkaline phosphatase concentrations overlooked. When pharmacologic intervention for low bone density is considered, patients with unexplained low alkaline phosphatase should undergo clinical, biochemical, and possibly genetic assessment to determine if HPP is the cause. It is important to remember that HPP is a clinical diagnosis that should not be made based on biochemical or genetic testing alone. Not all individuals with a potentially pathogenic variant of *ALPL* will develop clinical features of HPP or demonstrate osteomalacia on bone biopsy.

Limited data are available regarding bone density changes and fracture outcomes with pharmacological treatment of adults with HPP. However, reports of successful treatment with anabolic therapy for low bone mass/fractures in adults with HPP have been published. Consensus is lacking regarding when enzyme replacement therapy with asfotase alfa is appropriate to treat adult onset HPP who have low bone mass and fractures. Mode of administration, monitoring schedule, side effect profile, and cost are barriers to its routine use in this population. Future work is needed to determine whether clinical features, biochemical parameters, bone histology findings, and/or genotype may provide clues to optimal therapy for adults with HPP and low bone mass.

Rheumatologic Manifestations of Hypophosphatasia (HPP)

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Patients with adult onset HPP present to rheumatology clinics with manifestations of metabolic bone disease, fractures, low bone mass, osteomalacia and with specific rheumatologic complaints. Those who present with low bone mass or fractures almost always have had years of diffuse musculoskeletal (MS) pain. MS symptoms are common in HPP and often a presenting symptom. In some series 60% of adults present with MS pain. In a series from Mayo Clinic, 68% of HPP patients diagnosed as adults were symptomatic and 41% (9 of 22) had MS symptoms.1 An EMR search for patients with persistently low alkaline phosphatases (ALP) from Marshfield Clinc, 69% had MS symptoms defined as a diagnosis of a primary rheumatic disease, or symptoms sufficient to warrant specialty consultation.2 In a series of 42 patients with persistently low ALP, 24 had skeletal and muscular pain.3 In a series of 84 patients with low ALP, the odds ratio for MS pain was 7.6 in those with pathogenic ALPL variants versus those without.4

Defined MS manifestations include chondrocalcinosis, enthesopathies, calcific periarthritis, diffuse idiopathic skeletal hyperostosis and exostoses. Chondrocalcinosis, a radiologic diagnosis, is likely the result of increasing concentrations of PPi, calcium and phosphorus available in the tissue microenvironment. The clinical sequelae of chondrocalcinosis, calcium pyrophosphate deposition disease (pseudogout) is less common.

The most common MS manifestation is widespread, non-specific pain in muscles, bones, joints and periarticular tissues. Fatigue both generalized and muscular are common complaints. This presentation has been termed the pain phenotype. These symptoms often lead to a diagnosis of fibromyalgia in many patients. Radiologic surveys in these patients may be negative but may show perarticular calcifications around the shoulders, knees, hips and other locations. The presumed cause of the pain is underlying metabolic bone disease, hypomineralization and osteomalacia. Treatment with asfotase alfa often but not always improves symptoms but requires months of treatment for improvement.

1. Berkseth KE, et al. Bone 2013;54: 21-27
2. McKiernan FE, et al. J Bone Miner Res 2014;29: 1651-1660
3. Riancho-Zarrabeitia L, et al. Eur J Int Med 2016;29:40-45
4. Tornero C, et al. Orhpanet J Rare Ds 2020;15:51-60

HPP - What Have We Learned? Indications for Treatment?

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One of the most important lesson learned in HPP over recent years is the simple truth that we don’t know enough. Coming a long way from an ultrarare skeletal dysplasia, we're now getting more and more aware that HPP isn’t just a bone disease but rather a complex condition marked by generalized, episodic pain, exhaustion and fatigue and prolonged recovery upon exercise. Further manifestations appear to involve the neurological system with headache / migraine , so-called brain fog and peripheral dysesthesia as well as GI issues, altogether causing compromised physical performance and substantially affecting quality of life.

Along with that we will have to learn that not all of the manifestations we’re facing now can be attributed to elevated PPi and PLP, and other pathophysiologic mechanisms previously proposed have to be scrutinized to better understand the causality of distinct signs and symptoms as a prerequisite for targeted treatment strategies.
Medical treatment should be made available to everyone who can substantially benefit without a disproportionate risk. For the compound approved for HPP now, we have pretty clear data, what manifestations and accordingly what patients can (sustainably) profit from that treatment. This is first in line those with bone manifestations which is typically the case in subjects with biallelic variants and early and overt pediatric disease manifestation, no matter at what age they are eventually diagnosed.

For all others, treatment is currently limited to supportive therapies and concepts. Considering the perspective on the disease given above, we will have to identify, develop and evaluate agents and treatment modalities that reflect not only the ultrarare skeletal dysplasia but also all the other manifestations impeding patients’ quality of life.

Insights into dentoalveolar defects associated with hypophosphatasia from novel mouse models

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Hypophosphatasia (HPP) is caused by loss-of-function mutations in *ALPL*, which encodes tissue-nonspecific alkaline phosphatase (TNAP). In addition to rickets and osteomalacia, HPP is associated with a range of defects affecting oral health and manifesting as premature tooth loss and periodontal disease. The *Alpl* knockout mouse model of severe HPP exhibits early lethality that limits dental studies. We aimed to use novel mouse models of HPP to study unanswered questions regarding mechanisms of dentoalveolar defects and treatment effects on these tissues. We crossed mice carrying a floxed *Alpl* allele with Cre recombinase harboring lines to conditionally ablate *Alpl* in ectomesenchymal tissues (using *Wnt1-Cre2*) and epithelium (using *Krt14-Cre*).

Dentoalveolar tissues harvested from control and conditional knockout (cKO) mice at 2 months were analyzed by micro-computed tomography, electron microscopy, and histological approaches. *Wnt1-Cre2; Alplfl/fl* cKO mice eliminated *Alpl* from dentin/cementum/bone-associated cells. Compared to controls, these cKO mice showed no difference in body weight or appendicular bones, but had significantly reduced circulating ALP. Compared to controls, cKO molars exhibited severely affected dentin and alveolar bone, lack of acellular cementum, and detachment of periodontal ligament. *Krt14-Cre2; Alplfl/fl* cKO mice deleted *Alpl* from the enamel organ. These cKO mice showed no difference from controls in body weight or appendicular bones and no change in ALP.

Compared to controls, these cKO mice displayed visible enamel defects in males, including discolored and rough surfaces, reduced density and volume, altered microstructures, and detachment, aberrant proliferation, and disorganized ameloblasts. In summary, conditional ablation of *Alpl* in mice recapitulated key dentoalveolar defects of HPP, providing new models for study. The dentin/cementum/bone cKO mice provide a model for longitudinal testing of treatment effects. The enamel cKO mice showed surprising sex-associated differences and revealed that enamel is directly affected by TNAP loss-of-function. Other Cre-drivers will allow additional studies on multisystem effects of HPP.

Compromises in skeletal muscle and myoblast energetics are associated with compromised kinematics in sheep with hypophosphatasia (HPP)

Joshua Bertels, Alyssa Falck, Constance Vernham, Shannon Huggins, Danilo Landrock, Chloe Guy, Jordan Ankersson, Aaron Henry, Erin Giles, Charles R Long, Sarah H White-Springer, Michael Moreno, Larry J. Suva, Dana Gaddy.

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Hypophosphatasia (HPP) is a rare inherited disorder of mineral metabolism, the result of inactivation of the tissue-nonspecific alkaline phosphatase (TNSALP) gene (*ALPL*) that is characterized by decreased bone and tooth mineralization, premature tooth loss and muscle weakness in humans. Based upon a HPP patient, an Isoleucine -> Methionine (c.1077 C>G) mutation was introduced into the Rambouillet sheep *ALPL* gene using Crispr/Cas9 gene editing. HPP sheep recapitualte diminished mineralized bone and dental deficiencies like humans with HPP. Kinematic analysis of HPP and WT sheep demonstrated spinal sway as well as reduced stride velocity. To determine the etiology of muscle weakness, HPP and WT skeletal muscle was assessed for changes in myofiber size and fiber type. Results showed reduced oxidative fibers in gluteal muscle of HPP sheep, as well as reduced myofiber size, regardless of fiber type. Mitochondrial size was significantly reduced in gluteal muscle, suggesting reduced mitochondrial biogenesis. High resolution respirometry of permeabilized muscle fibers demonstrated reduced oxidative capacity and coupling efficiency. Similarly, at the cellular level, myoblasts from HPP sheep showed delayed myotube differentiation and reduced coupled (ATP-linked) and non-coupled maximal respiration rates compared to WT by Seahorse analysis. Collectively, these data demonstrate that HPP sheep have compromised gait kinematics, reduced myofiber and mitochondrial size, and reduced oxidative respiration and glycolysis in sheep skeletal muscle. These deficiencies at the tissue level mirror the deficiencies seen in myoblast cellular respiration during their delayed differentiation and provide a mechanistic basis for the reduced myofiber size observed in HPP sheep muscle. These cellular data provide evidence that the altered gait and slower stride observed in HPP sheep is associated with reduced fiber growth due to impaired bioenergetic capacity, and demonstrate a fundamental requirement for TNSALP activity in the maintenance of normal cellular respiration in cells of the musculoskeletal system.

Fat Metabolism in Hypophosphatasia: Clinical and Basic Implications

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Besides the very severe skeletal manifestations of hypophosphatasia, other organs and tissues exhibit changes that contribute to impaired quality of life and morbidities in adult life. We and others have previously noted that TNAP (tissue non-specific alkaline phosphatase) is expressed in high levels not only in osteoblasts, but also in adipocytes, with inhibition of TNAP activity leading to decreased lipid accumulation. However, the function of TNAP in fat cells remains to be determined.

Thus, we have been utilizing various in vivo genetic models of hypophosphatasia to more clearly identify the function of TNAP in adipocytes. Global *alp-/-* mice have very short lifespans, exhibit skeletal manifestations of HPP along with reductions in body weight, fat mass and adipocyte size. Pro-adipogenic and fat metabolism genes are markedly suppressed in mesenchymal stromal cells isolated from these mice with fewer adipocytes noted during differentiation. Importantly mitochondrial function studies revealed impaired oxidative phosphorylation, a critical step for ATP generation and energy production.

Our lab recently generated PAKO mice, *Prrx1-Cre-Alplfl/ko,* a mesenchymal specific genetic deletion of *Alpl,* which may better recapitulate adult HPP phenotypes. *PAKO* mice exhibit skeletal defects with less bone marrow adipocytes compared to controls. In addition, these mice have smaller inguinal fat mass, adipocyte size and lower whole body energy expenditure. Mitochondrial function was also reduced in both *PAKO* bone marrow and mesenchymal stromal cells. Taken together, these lines of evidence support the hypothesis that hypophosphatasia is a systemic disorder, characterized by changes in both the osteoblastic and adipogenic pathways beyond mineralization phenotypes. The defective mitochondrial function and energy expenditure noted with TNAP deficiency may be causative of chronic fatigue phenotypes found in adult HPP patients. Clinical studies are needed to assess changes in body composition and energy expenditure in hypophosphatasia to link these observations from the bench to beside.

Hypophosphatasia and Ectopic Calcification: Ocular Findings From the Enobia Study

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Introduction: Hypophosphatasia (HPP) is a rare, metabolic disorder that can result in abnormal mineralization of bone and teeth. The severity ranges from mild to severe and appears to be dependent on both age of onset and inheritance pattern. There are currently six classifications of HPP. In 2010, Enobia Pharmaceuticals initiated a study to determine the effectiveness of an experimental enzyme replacement drug known as asfotase alfa (AA). One critical aspect of this study involved the observation and monitoring of abnormal calcium deposition in the eye.

Methods: A total of 13 patients (mean age 10.92 years; range 6 -16 years) were enrolled at St. Louis Children's and Shriner's Hospitals between April 2010 and August 2016. All thirteen of these patients were placed on AA. Ophthalmologic examinations included the following: visual acuity, ocular motility, intraocular pressure, biomicroscopy, and indirect ophthalmoscopy. Patients were seen every six months and had dilated fundus exams annually. We were primarily concerned with the development of abnormal calcium deposition, optic atrophy, and angioid streaks of the retina.

Results: Nine patients (69%) developed evidence of calcium deposits involving the cornea and/or conjunctiva. Four patients (31%) exhibited no signs of ectopic calcification. Five of the patients with ectopic calcification (55.6%) were diagnosed prior to startingenzyme replacement therapy, while 4 patients (44.4%) were diagnosed after starting enzyme replacement therapy. Two patients (15%) were diagnosed with optic atrophy secondary to hydrocephalus and none of the patients developed angioid streaks of the retina.

Conclusion: It is clearly evident that HPP can cause ectopic calcification of the eyes. It is possible that treatment with AA may do the same, but further study of this issue would be necessary to arrive at such a conclusion. It is important to note that no patient suffered vision loss as a result of ectopic calcification.

Are there Phenocopies of HPP? (Yes!)

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Hypophosphatasia (HPP) is increasingly understood to have an extremely wide variability in presentation. Phenocopies of HPP, which may present with similar clinical and radiological features as HPP, can be caused by other genetic and acquired conditions. Because of the extreme variability in the presentation of HPP, the number of partial phenocopies is vast, but this presentation will focus on a few key conditions.

An important and increasingly appreciated phenocopy of HPP is the hypermobility form of Ehlers-Danlos syndrome (hEDS). Unlike in HPP, the etiology of hEDS remains largely unknown. Patients with hEDS present with joint hypermobility, chronic pain, and connective tissue fragility. Some patients with hEDS have been described with low bone mass and a propensity for fracture, which can mimic the bone manifestations seen in HPP. The potential similarities highlight the importance of genetic testing in differentiating between HPP and its phenocopies. Another genetic phenocopy of HPP is osteogenesis imperfecta (OI), which may overlap with HPP in causing bone fragility low bone mass, and hypermobility.

Fibromyalgia and osteoporosis are acquired conditions that can present with clinical features resembling HPP. Like HPP, fibromyalgia can cause diffuse musculoskeletal pain, fatigue, and sleep disturbances, while osteoporosis is characterized by low bone mass and an increased risk of fractures. Hypothetically, neither of these acquired conditions is exclusive of a diagnosis of a genetic condition such as HPP.

In conclusion, phenocopies of HPP can present diagnostic challenges, as they can mimic the clinical and radiological features seen in HPP. Genetic testing can be helpful in differentiating among HPP and its phenocopies. Recognition of these phenocopies can aid in the appropriate management and treatment of affected individuals.

Biochemical Assessment of Adults in the Metabolic Bone Disease Clinic

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The two key biochemical tests of most value in the diagnosis of hypophosphatasia (HPP) in adults are alkaline phosphatase activity (ALP) and pyridoxal 5'-phosphate (PLP). The most useful form of ALP is total enzyme activity (it is not necessary to measure the bone isoform). The world has moved towards standardisation of ALP assay, although there are still some reports in the literature of older reference intervals. The most used method has a reference interval of around 35 to 120 IU/L. Not all patients with clinically evidenced HPP have values of ALP below 35, and so I prefer to use the concept of a threshold and use a value of 43 IU/L. The second key biochemical test is PLP, a form of vitamin B6, which is usually measured by HPLC. The level is influenced by the use of vitamin supplements (high) and inflammation and chronic kidney disease (low) and so the presence of these conditions needs to be considered.

We had established reference intervals for men and women (men are higher), Mexican Americans, White non-Hispanic and Black non-Hispanic from the NHANES surveys in large numbers of subjects (over 4000) and find the upper limits of the reference interval do vary. In White British, we find a value of 120 nmol/L a useful cut-off. The careful use of ALP and PLP assays means that in most cases of suspected HPP we can be confident about the diagnosis, but in some of the less clearcut cases we would recommend genetic testing, too.

Orthopedics for Pediatric Hypophosphatasia

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The presentation of children with hypophosphatasia to the pediatric orthopedist is changing. A review of the children followed by the Children's National Hospital (CN) Bone Health Program underscores both improving recognition of the disorder and changing management. Historically children with HPP might present with bowing and concern for fracture. Today, many of these children are identified in utero, leading to amniocentesis, molecular diagnosis, and early treatment. Replacement enzyme straightens the bowing without orthopedic intervention. Of note, however, two children followed at CN also have elbow abnormalities: one with radio-ulnar synostosis, and one with radial head dislocation. In our experience, unexplained pain has been the most common presentation of juvenile onset HPP.

One child became wheelchair dependent due to extremity pain and weakness before his diagnosis was made. Another was mis-diagnosed as having CRMO based on frequent falls and multiple lesions on MRI. Yet another teenager had a long history of psychologically debilitating pain, early failure to thrive and multiple medical complaints. Other presentations include a patient identified on routine lab exam whose genu valgum is improving with asfotase alpha, and two siblings with periodic fevers who are currently not on therapy but are being monitored. Only one child has presented because of deciduous tooth loss. On-going management challenges include injection site problems, insurance denials, and case finding, because the ICD-10 code 83.39 is frequently mis-used. Multiple osteotomies with intramedullary fixation was occasionally recommended to treat significant bowing deformity in the past, but is no longer needed with early initiation of enzyme therapy.. Careful genetic exploration has profound implications for the whole family. Most of the children had a parent with the same mutation. Children with HPP benefit from a multidisciplinary approach involving Genetics, Endocrinology, and Orthopaedics.

Transition of Pediatric to Adult Care: Challenge and Opportunity

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Improving healthcare transition (HCT) from pediatric to adult medical systems is a Strategic Plan 2020 priority for National Institute of Child Health and Human Development (NICHD) encompassing all chronic diseases. Hypophosphatasia (HPP) presents unique challenges arising from its rarity and the novelty of enzyme replacement therapy (ERT). As pediatric management of HPP has evolved from supportive care to treatment, the emerging evidence base has several gaps which make HCT challenging. ERT created novel HPP phenotypes which did not exist 15 years ago, "treated infantile and juvenile HPP".

In childhood, HPP is a disease of growing bones, "rickets". The main treatment objective is correcting deficient mineralization at cartilaginous growth plates to build a functional musculoskeletal system. This would seem to reach an endpoint when the epiphyses fuse in adolescence. In adults, HPP is a disease of abnormal osteoid mineralization, osteomalacia, literally leading to "soft bones". The main treatment objectives become maintaining function, preventing fractures, and managing pain. Pediatric and adult phases of HPP require different multidisciplinary teams. We will look to the HCT experience with Osteogenesis Imperfecta (OI) as a model. The best practices in HPP management in different healthcare settings will be surveyed. The central role of patients and support organizations in developing an HCT strategy will be emphasized. Opportunities to create the evidence base and infrastructure to support HCT in HPP will be outlined and discussed..

Enzyme Replacement Therapy Cessation in Adults with Hypophosphatasia

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The burden of illness in patients with hypophosphatasia (HPP) is now well documented. However, there are limited data currently available regarding outcomes following discontinuation of enzyme replacement therapy (ERT) with asfotase alfa. The aim of this presentation is to present Real World Evidence regarding the impact of discontinuing ERT in adult patients with HPP. Four cohorts of patients will be presented. One cohort of adults with HPP who participated in the clinical trial of asfotase alfa, stopped treatment for a period of 18 months after the trial ended and then restarted ERT. Deterioration of disease burden off treatment followed by a rescue of symptoms with reinitiating of ERT will be reviewed.

This will be compared with three additional cohorts, a second cohort of adult patients with HPP not in a clinical trial and who discontinued treatment due to perceived lack of efficacy, a third cohort who started ERT but discontinued treatment due to lack of access and finally a fourth cohort of patients who discontinued ERT due to hypersensitivity reactions including anaphylaxis and/or intolerable injection site reactions and who remain off ERT. Description of disease burden and other demographics will be compared in these groups to evaluate if certain disease features might predict compliance or tolerance of ERT. Finally important questions of equity in who can access ERT and potential solutions will be discussed.

Monitoring of Physical Performance in Patients with Hypophosphatasia

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Standardized tests and outcome measures (STOM) are essential tools in the evaluation and treatment of rare metabolic bone disease at baseline and throughout the continuum of care. Outcomes can successfully identify functional deficits, support the decision for treatment, monitor effectiveness, and identify red flags in population of individuals with hypophosphatasia (HPP). In the initial research of pharmaceutical treatment of HPP, several STOM were identified and validated in the HPP population to quantify observations, monitor treatment, and document changes in function. Barriers can exist in the continued use of STOM in a clinical setting due to the need for additional resources, collaboration, and organization, especially when multiple providers and varying medical record systems are part of a patient's comprehensive care.

Practitioner use of a standard data collection tool can improve accuracy and productivity by guiding the consistent collection of specific tests and measures over the course of care. Data will then be available for use in ongoing evaluation and comparison, or retrospective study. A sample pediatric data collection tool will be presented that guides consistent evaluation using specific validated outcomes based on the patients age and function. Evidence from three case studies will be presented to demonstrate the use of outcomes in monitoring the effect of treatment, identify changes in patient status, and alert practitioners in the need for redirection or reeducation on treatment methods.

Hypophosphatasia Pregnancy? (Prenatal Treatment for Baby?) - Possibility of prenatal gene therapy for lethal HPP model mice

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Hypophosphatasia (HPP), caused by mutations in the *ALPL* gene encoding tissue-nonspecific alkaline phosphatase (TNALP), is an inherited systemic skeletal disease characterized by mineralization defects of bones and teeth. HPP model mice (*Alpl*-/-) phenotypically mimic the severe infantile form of human HPP; they appear normal at birth but die by 3 weeks of age because of growth failure, hypomineralization, and epileptic seizures. We have demonstrated that a single injection of adeno-associated viral (AAV) vector expressing bone targeted TNALP-D10 into postnatal HPP mice resulted in prolonged seizure-free survival and phenotypic correction. Because of the remarkable progress in prenatal diagnosis with clinical imaging, including echography and computed tomography, as well as molecular testing, the chance of diagnosis of perinatal lethal HPP during the fetal period is increasing. Therefore, prenatal gene therapy may be among the treatment options for perinatal and severe infantile HPP in the future. To investigate the possibility of prenatal gene therapy using the lethal HPP model mice, the fetuses of HPP model mice (on day 15 of gestation) underwent transuterine intraperitoneal injection of AAV serotype 9 vector expressing TNALP-D10. Treated and delivered mice showed normal weight gain and seizure-free survival for at least 8 weeks. Vector sequence was detected in systemic organs including bone at 14 days of age. ALP activities in plasma and bone were consistently high. Enhanced mineralization was demonstrated on X-ray images of the chest and forepaw. Our data clearly demonstrate that systemic injection of AAV9 in utero is an effective strategy for the treatment of lethal HPP mice. Prenatal gene therapy may be an important choice after prenatal diagnosis of life-threatening HPP.

Hypophosphatasia Pregnancy? (Prenatal Treatment for Baby?)

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During the third trimester of healthy human pregnancies, maternal circulation manifests a substantial increase in alkaline phosphatase (ALP). This is primarily from appearance of the placental ALP isoenzyme (PALP) detectable in the mother's serum, but also detectable within the cytoplasm and on the surface of her blood neutrophils. In HPP before pregnancy there is low circulating tissue-nonspecific alkaline phosphatase (TNSALP) in serum, and also in blood neutrophils. During both healthy or HPP pregnancies, beginning at week 12 of gestation, PALP is synthesized in placental syncytiotrophoblast, and is then shed into the maternal but not fetal circulation. PALP is expressed from the fetal genome, yet the fetus does not harbor this PALP. The maternal circulating PALP level increases as the placenta grows, but abruptly returns to baseline after the placenta is delivered. Does this PALP impact human HPP pregnancies directly in the mother and indirectly in the fetus? We have reported that PALP is catalytically active toward PEA, PPi, and PLP as shown during the third trimester of women considered carriers of HPP who substantially decreased their endogenous accumulation of these natural substrates for ALPs (Whyte, et al: Journal of Clinical Investigation 95: 1440-1445, 1995). Apparently unknown is whether women with overt osteomalacia from HPP improve their skeletal mineralization during a pregnancy. We do not have non-decalcified transiliac HPP biopsy data reflecting pre- and immediately post-pregnancy bone. Improvement would not be from soluble PALP, which is non-functional, but because PALP replete neutrophils and "maternal facing" placenta are rich with physiologically active PALP. Does this functional ALP somehow explain the dramatic third-trimester *in utero* improvement sometimes encountered in severe HPP? Perhaps also then resulting in instances of "benign prenatal HPP" featuring further improvement after delivery? In benign prenatal HPP, the fetus seems adversely impacted by the metabolic environment of a carrier or affected mother. Does the fetus benefit indirectly from the circulating PALP correcting ALP substrate accumulation in the mother? Phosphorylated compounds seem not to cross the maternal-fetal placental barrier. In mice, bone-targeted recombinant ALP given prenatally to heterozygous pregnant carrier female HPP mice and continued for their homozygous newborn pups with severe HPP did not seem to provide prenatal benefit. However, mice do not have a gene specifically for PALP. Can severe human HPP *in utero* benefit from maternal enzyme-replacement treatment?

Gene Therapy for Hypophosphatasia

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Enzyme replacement therapy with asfotase alfa (Strensiq) has proven lifesaving and greatly improves the quality of life in patients affected by the most severe forms of the disease. However, the almost daily injections of this protein therapeutic often cause injection site reactions sometimes severe enough to force discontinuation of treatment which results in deterioration of the physical condition of the patients. Work towards devising an alternative viral vector-mediated delivery of this therapeutic started already after the first meeting of Hypophosphatasia Europe, in May 2007, Huningue, France, when the first pre-clinical data of efficacy of asfotase alfa in the mouse model of infantile HPP (*Alpl-/-* mice) was presented. In attendance at that meeting was gene therapy expert Professor Takashi Shimada (Nippon Medical School, Tokyo, Japan), who saw a path forward towards the development of viral-mediated delivery of mineral-targeted alkaline phosphatase (TNAP-D10), so that the body's own cells would continuously produce TNAP-D10, rather than requiring daily injections of a protein produced and purified *ex vivo*. The collaboration between Professor Shimada and his now successor Professor Miyake and the Millan laboratory has produced to-date 15 publications exploring the pre-clinical efficacy of a single injection, either systemically or locally, of different types of viral vectors with different levels of expression and tissue distribution.

Two papers in 2021 documented that a single injection of adeno-associated virus serotype 8 encoding TNAP-D10 led to preservation of life, and prevention of the skeletal and dental phenotype characteristic of the severe mouse model of infantile HPP (Matsumoto et al., 2021, DOI: 10.1016/j.omtm.2021.06.006; Kinoshita et al., 2021, DOI: 10.1002/jbmr.4382). More recently, this AAV8-TNAP-D10 vector also proved efficacious in ameliorating skeletal and dental disease in a mouse model of late-onset HPP and in a mouse model of PHOSPHO1 deficiency, a related soft bones condition that manifests as pseudo-HPP (Amadeu de Oliveira et al., 2023, DOI: 10.1002/jbm4.10709). Currently, the Miyake and Millan laboratories are collaborating in performing investigational new drug (IND)-enabling studies aimed at bringing this gene therapy approach to clinical trials. Gene therapy as a treatment modality promises to greatly reduce the injection burden in HPP patients and possibly allow milder forms of the disease to qualify for treatment.