Chapter

Gene Therapy for Hypophosphatasia: Current Management and Future

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Abstract

This review provides a comprehensive overview of hypophosphatasia (HPP), a rare genetic disorder marked by defective bone and teeth mineralization due to mutations in the *ALPL* gene. It reviews the evolution of HPP treatment, from early symptomatic management methods to the latest therapeutic approaches, emphasizing significant milestones achieved over time. In addition, this review delves into gene therapy's historical development, outlining its successes and challenges. Furthermore, it presents a detailed analysis of why this innovative therapy holds promise for HPP, focusing on its efficacy in correcting the underlying biochemical defects and significantly improving patient outcomes. Moreover, the review discusses future research directions, highlighting the critical need for ongoing innovation and rigorous clinical trials to further enhance the efficacy and safety of gene therapy for HPP. Continuous research is essential to developing more effective treatments and ultimately improving the quality of life for patients affected by this debilitating condition.

Keywords: Hypophosphatasia, alkaline phosphatase, therapeutics, progress, genetic therapies, adeno-associated virus vector

1. Introduction

Hypophosphatasia (HPP) is a rare genetic disorder characterized by defective mineralization of bones and teeth due to low activity of the tissue-nonspecific alkaline phosphatase (TNAP) isozyme, encoded by the *ALPL* gene. The severe forms of HPP primarily exhibit autosomal recessive inheritance, whereas the milder forms predominantly exhibit autosomal dominant inheritance. Symptoms vary widely from embryonic to adult onset, depending on the levels of residual alkaline phosphatase (ALP) activity. The disorder is classified into six types: perinatal, benign prenatal, infantile, childhood, adult, and odontohypophosphatasia [1, 2].

Inorganic pyrophosphate (PPi), a calcification inhibitor, is typically hydrolyzed by TNAP. Elevated PPi levels in the plasma of patients with HPP prevent hydroxyapatite mineral precipitation and growth. Another substrate of TNAP is pyridoxal-5'-phosphate (PLP), which is essential for a wide range of biochemical reactions,

including neurotransmitter synthesis [3]; its deficiency in the brain can cause vitamin B6-dependent convulsions [2]. In addition, urinary phosphoethanolamine (PEA) levels are elevated in patients with HPP, aiding in the diagnosis of HPP. However, the clinical significance of PEA levels and the affected metabolic pathways remain unclear.

The incidence of severe HPP is reported to be 1/100,000 people in North America [4], 1/300,000 people in Europe [5], and 2–3/1,000,000 people in Japan [6, 7], whereas the nonlethal forms are more prevalent. Actual prevalence of HPP is possibly higher than previously reported, as clinical symptoms are less prominent in certain individuals, while the same mutations cause severe phenotype in other patients. Additionally, many patients with minor illnesses may remain undiagnosed [8, 9]. For example, the del1559 mutation in *ALPL* is frequently observed in Japanese population, and its carrier incidence is approximately 1 in 480 individuals [8]. Adult-onset HPP often presents with nonspecific symptoms, such as chronic pain and osteoporosis-like radiographic findings, leading to frequent misdiagnosis and may go undetected because of inaccurate diagnosis [10–12]. The number of patients with HPP may be higher than previously reported, as some cases are asymptomatic but have genetic mutations in TNAP or heterozygous genetic mutations [8, 13]. Furthermore, over 400 mutations of the *ALPL* gene have been reported [14, 15]; these mutations result in a spectrum of clinical manifestations, ranging from mild to severe skeletal abnormalities.

As a single-enzyme-deficient disease, HPP is suitable for therapeutic interventions. Asfotase alfa, a recombinant enzyme replacement therapy (ERT), became available in 2015 [16] and revolutionized HPP treatment. However, ERT requires frequent injections throughout a patient's life, posing a burden, particularly in pediatric cases with early onset of the disease [17]. The success of ERT has propelled gene therapy to become the next frontier in HPP treatment. In this chapter, we review the history of HPP treatment, evolution of gene therapy, ongoing gene therapy research on HPP, and prospects for future treatments.

2. Pathophysiology

In children with HPP, serum ALP levels are significantly lower than age-matched normal levels. The reference range for serum ALP varies with age and is typically higher in children due to active bone metabolism during growth [18]. Thus, ALP values that are usually normal in adults may fall below the normal range in children, often leading to misinterpretation and delays in diagnosis and treatment. Therefore, age and sex must be considered when assessing ALP levels to avoid overlooking HPP cases [19].

Furthermore, HPP in pregnancy requires caution. Owing to the presence of placenta-derived ALP, serum ALP activity is generally higher in pregnant than in nonpregnant women. Thus, low plasma ALP activity could be normal or high during pregnancy, masking a physiologically low bone-derived TNAP [20, 21].

The *ALPL* gene, recently renamed "alkaline phosphatase, biomineralization associated" [22], encodes TNAP and is located at 1p36.12 on the short arm of chromosome 1. More than 400 mutations have been reported associated with HPP [14, 15], and the genotype and phenotype do not always correlate. Patients with identical genotypes may exhibit variations in the timing of disease onset and symptom presentation. Predicting symptom onset and presentation in later-born children, even if they share the same genetic mutation as earlier-born children in a family

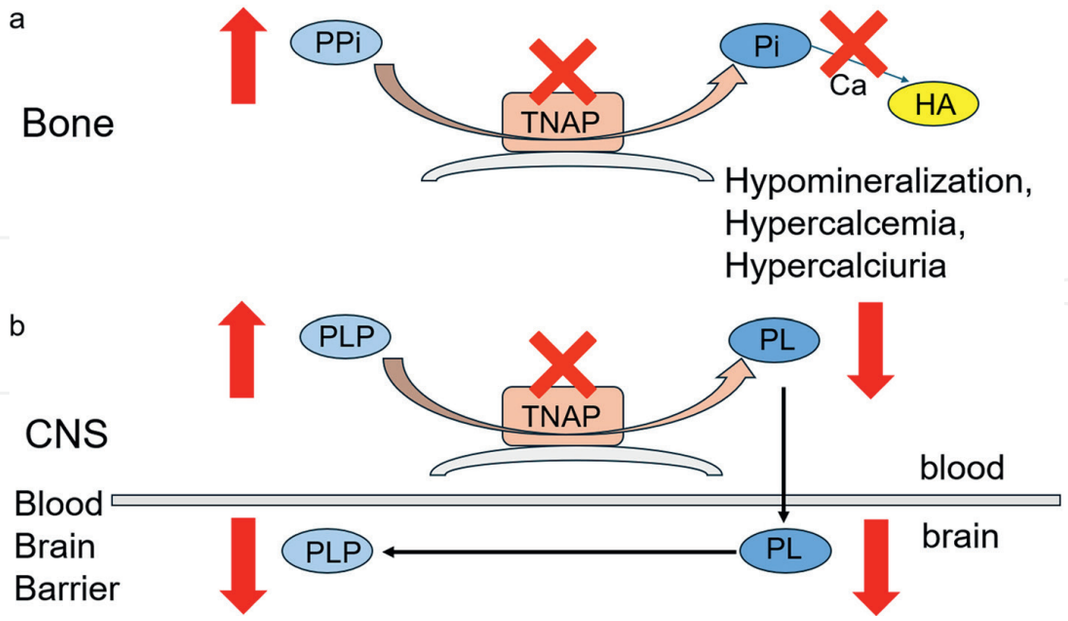

Figure 1.
Mechanisms of tissue nonspecific alkaline phosphatase (TNAP) in bone mineralization and brain function: In the bone, TNAP functions as a pyrophosphatase in bone tissue, producing phosphate (Pi) from pyrophosphate (PPi), which binds to calcium and deposits on hydroxyapatite, facilitating ossification (a). In the brain, TNAP functions as a pyrophosphatase in the blood converting pyridoxal 5′-phosphate (PLP) to pyridoxal (PL). Only PL crosses the blood–brain barrier, where it is reconverted to PLP to act as a neurotransmitter metabolism coenzyme (b).

linkage, remains a challenge [1, 5, 23]. Diminished TNAP activity leads to accumulation of TNAPs substrates including PPi, PLP, and PEA. Besides, TNAP is known as the liver-bone-kidney type ALP because it is abundantly expressed in those tissues. However, it is also extensively distributed throughout other organs. Other notable tissues that may also contribute to serum ALP activity through the expression of distinct ALP encoding genes (*ALPI, ALPP, ALPPL2*) include the small intestine, placenta, and germ cells.

In the body, PPi binds directly to hydroxyapatite crystals, thereby inhibiting ectopic calcification of soft tissue. In contrast, in the skeleton and teeth, TNAP hydrolyzes PPi to inorganic phosphate, thereby promoting crystal growth. The resulting phosphate binds to calcium to form hydroxyapatite; thus, the calcium that is not utilized for bone formation remains in the bloodstream, leading to hypercalcemia and hypercalciuria (**Figure 1a**).

Furthermore, PLP is involved in the synthesis of neurotransmitters in the brain. Because PLP cannot cross the blood–brain barrier, it is first dephosphorylated by TNAP to pyridoxal (PL). After crossing the blood brain barrier, PL is re-phosphorylated in the brain to PLP for utilization as an active cofactor of glutamate decarboxylases (**Figure 1b**). Thus, decreased TNAP activity is associated with reduced PLP levels in the brain, leading to vitamin B6-dependent convulsion [24–26].

3. Clinical presentation

Typically, HPP is classified according to the time of onset and severity, as presented below. However, as the disease progresses, the symptoms may increase, leading to a change in diagnosis [27]. Moreover, diagnosis is challenging because the phenotype does not always correlate with the type of genetic variant [5].

3.1 Perinatal hypophosphatasia

Typically, perinatal HPP [28] is fatal because of recurrent *in utero* fractures and respiratory complications arising from decreased thoracic circumference malformations, regardless of normal trunk length [29–31], as is shown in **Figure 2** [32]. However, there has been a paradigm shift with the advent of ERT, which has significantly improved survival rates and outcomes in patients with perinatal HPP. Perinatal HPP usually results from homozygous or compound heterozygous mutations of the *ALPL* gene, following an autosomal recessive inheritance pattern. On radiographs, it is evident that bone calcification manifests randomly [23]. For instance, some vertebrae may display calcification, whereas other vertebrates may not. In the skull, the frontal bone typically undergoes initial ossification; among the bones of the body, the clavicle represents the initial ossification site. Nevertheless, bones throughout the body are typically sufficiently thin to avoid detection on radiographs. Symmetrical defects in metaphysis of long bones such as femurs are characteristic of HPP, a condition referred to as central "tongue" [33].

Bowdler spurs represent a prevalent osseous characteristic of HPP [34–38]; they manifest as osteophytes projecting transversely from long tubular bones with deep skin dimples. However, the presence of Bowdler spurs alone does not warrant the exclusion of other diseases [35].

Osteogenesis imperfecta (OI) is more common [39] than HPP and should be differentiated based on the fetal bone abnormalities. OI manifests as a bone abnormality

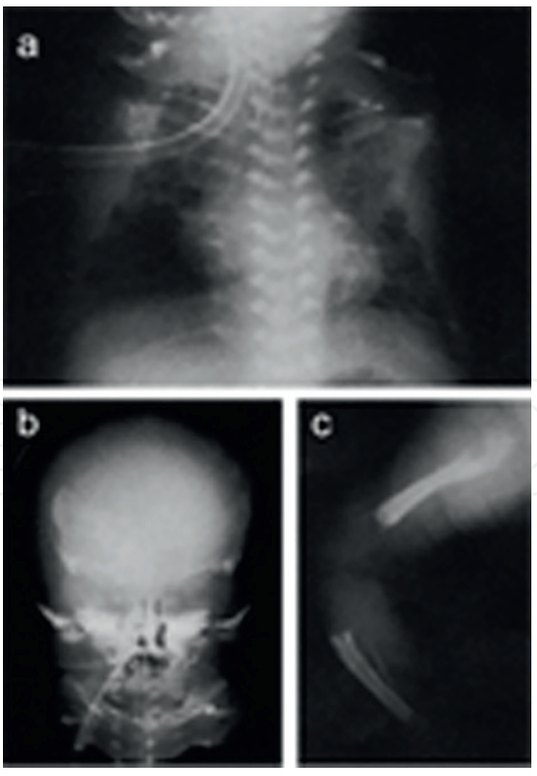

Figure 2.
Radiographs of a neonatal hypophosphatasia born at 42 weeks showing ossification failure and respiratory complications. Cesarean delivery was performed at 42 weeks, and 1-day gestation following fetal ultrasound showed extremity shortening. The baby received respiratory support from birth until death at 5 months due to respiratory failure. Clinical findings included vertebral ossification failure, and thoracic narrowing (a), skull ossification failure (b), and rickets-like changes in the femur (c). Reproduced with permission from Hirayama, T. et al.

during the fetal stage, and bisphosphonates are used to suppress bone resorption and improve ossification [40]. Conversely, HPP presents as a condition in which ossification is inherently impaired, rendering the application of bisphosphonates futile; thus, the use of bisphosphonates should be avoided [27]. Distinguishing these conditions is imperative, even in cases of prenatal bone anomalies.

3.2 Benign perinatal hypophosphatasia

Fetal echogenic bone abnormalities are detected prenatally, with a favorable prognosis characterized by improvement in bone abnormalities postnatally or in the third trimester of pregnancy [1, 5, 41]. Numerous studies on benign perinatal HPP [42] have been published in Japan, with some studies suggesting the effectiveness of treatment with asfotase alfa [43, 44]. However, treatment, particularly for rare diseases, is expensive and exerts pressure on the healthcare economy [45]. Ongoing discussions and research are crucially important to establish eligibility criteria for treatment among patients and explore potential alternatives for improved therapeutic outcomes [46].

3.3 Infantile hypophosphatasia

Infantile HPP [28] occurs within the first 6 months of life [1]. It usually results from homozygous or compound heterozygous mutations of the *ALPL* gene [27], following an autosomal recessive inheritance pattern. Craniosynostosis, severe skeletal abnormalities, hypercalcemia, hypercalciuria, and vitamin B6-dependent convulsion are the most common symptoms and are usually severe. Symptoms of infantile HPP are similar to those of perinatal HPP; however, the progression is comparatively slower, resulting in an extended lifespan for affected patients compared with those with perinatal HPP.

3.4 Childhood hypophosphatasia

HPP occurring after 6 months of age is typically classified as childhood HPP [47] and typically characterized by premature loss of fully developed teeth. It usually results from homozygous, compound heterozygous, or heterozygous mutations of the *ALPL* gene, following an autosomal recessive inheritance pattern. In addition, patients with childhood HPP frequently present with bone pain, muscle weakness, limb deformities, shortening, and dental symptoms. Furthermore, childhood HPP is characterized by tooth loss, primarily the front teeth, before age four, with the roots remaining intact [48, 49]. The median age at symptom onset in patients with autosomal recessive disease is 1 year, which is younger than that in patients with autosomal dominant disease (4 years). Blood levels of PPi and PLP were notably elevated in patients with recessively inherited conditions. However, clinical manifestations such as delayed ambulation and skeletal alterations were comparable between the two groups, although a higher incidence of bone fractures was observed among patients with the dominantly inherited disease [50]. Childhood HPP is nonfatal; however, it reduces quality of life and disrupts the ability to lead a typical childhood.

3.5 Adult-onset hypophosphatasia

Patients with adult-onset HPP [51] frequently complain of widespread chronic pain and muscle weakness [52, 53]; however, the pathophysiology remains unclear.

These patients may constitute undiagnosed cases of pediatric HPP; nonetheless, they often lack distinctive clinical features for identification. Symptoms, such as delayed fracture healing and those resembling osteoporosis, are commonly observed, along with pseudofractures and dysplasia of the bony bridges. Misdiagnosis as osteoporosis is frequent when HPP is not considered in the differential diagnosis [52, 54]. Bisphosphonates, one of the basic therapies for osteoporosis, is contraindicated in HPP [55, 56], making it crucial to include HPP in the differential diagnosis. Asfotase alfa has been shown to enhance bone mineralization and facilitate the healing of delayed union fractures in adult patients [57–61]. Moreover, ERT can restore muscle strength [62, 63]. Given the nonspecific symptoms observed in adult patients with HPP and the frequent misinterpretation of imaging results resembling osteoporosis, assessment of serum ALP levels is a valuable initial diagnostic approach [54]. In addition, a single diagnostic test is often inadequate. Notably, a substantial proportion of patients with milder forms of HPP show improvement with supportive treatments alone [64, 65]. However, a subset of patients requires ERT. Judicious selection of patients eligible for ERT poses an ongoing challenge in clinical management [66].

3.6 Odontohypophosphatasia

In odontohypophosphatasia, a notable feature is premature tooth loss at a young age, despite the absence of bone abnormalities [7]. The root of the fallen tooth remains intact, and pathology reveals cementum dysplasia. Typically, poor calcification of both the acellular cementum and dentin is observed [48]. However, it is challenging to prevent the losing of primary teeth upon initiation of ERT, which may commence within days of birth in cases of timely diagnosis. This difficulty arises because the primary teeth are formed during fetal development [67]. Therefore, absent teeth cannot be transplanted, necessitating denture placement. Although the mild variant of the condition has a more favorable prognosis than its severe counterpart, it nonetheless represents a significant detriment to the patient's quality of life. Cases initially diagnosed solely based on dental symptoms or considered odontohypophosphatasia may later be re-diagnosed as pediatric or adult-onset HPP after presentation of bone or muscle symptoms [49]. Studies indicate that ERT has positive effects on dental symptoms associated with infantile and childhood HPP [68, 69].

4. Treatment

Before the advent of ERT, only symptomatic management options were available. For severe perinatal HPP, treatment options were limited to ventilation and low-calcium milk for hypercalcemia, hypercalciuria, and vitamin B6-dependent convulsions [1]. Numerous modalities have been explored to address the needs of individuals with HPP. These interventions include bone marrow cell transplantation [70, 71], transplantation of bone fragments along with cultured osteoblasts [72], administration of teriparatide (recombinant human parathyroid hormone PTH 1–34) [73, 74], ERT utilizing ALP-enriched serum derived from patients with Paget's disease of the bone [75], and infusion of plasma sourced from healthy donors [76] or ALP purified from hepatic tissue [77]. Despite these interventions, noticeable clinical and radiographic enhancements have been observed only in some patients, reflecting the limited efficacy of these therapeutic modalities.

The clinical manifestations observed in TNAP knockout ($Alpl^{-/-}$) mice closely mimic those seen severe infantile HPP, making $Alpl^{-/-}$ mice an optimal animal model for studying HPP [78]. Although initially appearing phenotypically normal at birth, these mice progress to exhibit growth failure, vitamin B6-dependent convulsions, and hypomineralization, typically dying before weaning. $Alpl^{-/-}$ mice were rescued by daily subcutaneous administration of a bone-targeted variant of TNAP obtained by conjugating a bone-targeting deca-aspartate sequence to the C-terminus of soluble TNAP (TNAP-D_{10}) [79]. These promising preclinical outcomes paved the way for the clinical implementation of ERT in HPP patients [80]. This significant advancement has not only extended survival rates in patients with severe infantile HPP but also enhanced the quality of life for patients with pediatric and adult-onset HPP [81].

ERT significantly enhances prognosis, particularly for patients with severe perinatal HPP. However, as the enzyme degrades over time, continuous replenishment by subcutaneous injections 3–6 times a week is required [82]. Patients seek to alleviate the persistent pain due to the frequent injections and receive definitive curative therapy [83].

Furthermore, the use of ERT is associated with some challenges, such as the development of lipodystrophy at the site of injections and the development of antidrug antibodies [84]. Improvements in infantile HPP cases following bone marrow transplantation have been reported. In addition, allogeneic mesenchymal stem cell transplantation has demonstrated efficacy in fatal perinatal HPP [85]. However, the treatment is accompanied with pre-therapy procedures, such as chemotherapy, radiation therapy, and immunotherapy, and associated with potential posttransplant complications, such as graft-versus-host disease.

5. Gene therapy

5.1 History

Gene therapy was initially proposed as a radical treatment for diseases *via* repair of damaged genes. However, this modality proved challenging. In the early days of gene therapy, recognizing target genes was challenging, and gene modification techniques were insufficiently developed, making it difficult to alter target genes [86, 87]. Alternative approaches to deliver functionally missing molecules were necessary. Not all genetic diseases can be treated using gene therapy. However, gene therapy can serve as a potentially curative approach for diseases that meet two criteria: 1) the condition must be attributable to a single gene and 2) the expression of that gene can reach a therapeutic level.

The direct introduction of a functional gene into the body is known as *in vivo* therapy. The vectors employed for gene delivery include viral and nonviral vectors such as plasmid vectors, bacterial vectors, and lipoplexes. At present, the primary clinical application of viral vectors is therapeutic because of their high transduction efficiency. Although the use of viral vectors is simple and efficient, it requires a large viral dose and there is a possibility that the viral vectors may enter organs besides the target organs. Meanwhile, *ex vivo* therapy describes the process of cell collection, culturing, and transduction of functional genes prior to their administration to the patient. *Ex vivo* therapy is applicable in certain primary immunodeficiency diseases, congenital errors in metabolism, and other conditions that necessitate hematopoietic stem cell transplantation [88, 89]. CAR-T cell therapy [90], which exhibits antitumor

effects against refractory B-cell tumors, has been classified as an *ex vivo* therapy. It is expected to reduce side effects, such as secondary malignancy after CAR-T cell therapy, by allowing therapeutic cells to grow outside the body before returning to them and avoiding the transcription start site, thereby making gene therapy safer. Patient burden is substantial because of the necessity of pre-treatment before hematopoietic stem cell gene therapy.

In the years leading up to the 1990s, various attempts were made to use gene therapy for genetic diseases and cancer with the goal of achieving curative treatment. These attempts, however, had limited success. The first notable success was achieved in 2000 [91, 92]. Nevertheless, safety concerns arose a few years later because of incidences of leukemia development [93] and deaths resulting from other treatments [94, 95], thereby dampening public expectations. As a result of these limitations, advancements in techniques characterized by enhanced safety measures and ethical considerations have resulted in documented success since the late 2000s. For example, Zolgensma®, an *in vivo* gene therapy utilizing an adeno-associated virus (AAV) vector, significantly enhanced life expectancy and motor function in patients with spinal muscular atrophy, a condition previously lacking curative treatment. Although this represents a significant advancement in healthcare, the associated substantial expenses remain a significant challenge.

Studies have focused on genome-editing technologies, such as CRISPR-Cas9, to correct genetic defects caused by gain-of-function mutations in dominant genetic disorders [96]. CRISPR-Cas9 is a technology used for cutting and editing specific sections of DNA and has been clinically applied to treat sickle cell disease and beta-thalassemia. This method precisely targets and modifies faulty genes, thereby offering potential solutions for previously untreatable conditions. Casgevy® for beta-thalassemia [97, 98] was approved in Europe, the UK, and the USA. Extensive research and clinical trials are necessary to confirm the safety and efficacy of these techniques before wide application in clinical practice. In addition, CAR-T therapy involves genetically modifying a patient's T cells to recognize and attack specific cancer cells, primarily in blood cancers, such as multiple myeloma and lymphoma [99]. Kimliah® (CD19-CAR), Abecma® (BCMA-CAR), and nine other CAR-T therapy drugs have been approved. This therapy has been primarily applied in clinical settings for the treatment of blood cancer. Furthermore, oncolytic virus therapy involves the introduction of a natural or genetically engineered virus into the uterus, where it selectively replicates in the tumor tissue and targets tumor cells for destruction [100].

5.2 Challenges with gene therapy

Gene therapy faces several challenges, particularly regarding the use of different vectors. Local administration of AAV vectors is relatively safe, as seen with interventions like Luxturna® and Upstaza®, which require smaller doses and prevent significant immune responses. Luxturna® ($1.5x10^{11}$ vg/eye) is an intraretinal injection of voretigene neparvovec-rzyl [101] gene therapy drug, and Upstaza® ($1.81x10^{11}$ vg total) is an intraputamen injection of an AAV vector containing the human *AADC* gene (AAV2-hAADC) [102] gene therapy drug. However, systemic administration presents greater risks, including the requirement for large quantities of vectors. In cases of X-linked myotubular myopathy, the systemic administration of AAV8 at high doses results in fatal liver failure in some patients. At a low dose of $1.3x10^{14}$ vg/kg in seven patients and at a high dose of $3.5x10^{14}$ vg/kg in 17 patients, three patients from the high-dose group and one from the low-dose group experienced

fatal liver failure [103–106]. Similarly, in the treatment of Duchenne muscular dystrophy, high doses of intravenous recombinant AAV (rAAV) cause fatal immune responses and capillary leakage [107, 108]. The long-term sustainability and safety of AAV vectors remain uncertain, with the risks of inappropriate gene expression and immune responses. In addition, treatment efficacy relies on the absence of antibodies in the body that can neutralize AAV vectors (negative neutralizing antibodies), thus preventing treatment with AAV vectors [109].

Although advancements in gene therapy are underway, researchers must be mindful of uncharted areas that require exploration. To date, no study has established an association between systemic administration of AAV vectors and gene integration into germ cells. However, owing to the inability to examine all germ cells, a thorough confirmation of the absence of germ cell modification is essential [110, 111].

Lentiviral vectors, which integrate genes into random chromosomal locations, pose a risk of insertional mutagenesis, potentially leading to leukemia [112]. However, advancements in research have made self-inactivating (SIN) lentiviral vectors safer, eliminating the occurrence of leukemia. Recent clinical trials using SIN lentiviral vectors have brought them closer to clinical application [112, 113]. Other viral vectors can induce immune responses that diminish the efficacy of gene therapy and cause adverse effects. Although nonviral vectors pose fewer safety concerns and immune responses, they are associated with issues pertaining to efficiency and targeted gene delivery to specific tissues.

The high costs of gene therapy, particularly for rare diseases, pose a significant barrier. For example, Glybera®, a gene therapy for lipoprotein lipase deficiency approved in 2012, was discontinued in 2017 owing to low cost-effectiveness despite its high price [114]. The allocation of medical expenses for rare diseases is, therefore, heavily influenced by societal values and political policies. Gene therapy drugs such as LUXTURNA® for Leber congenital amaurosis, Zolgensma® for spinal muscular atrophy (SMA), and Hemgenix® for hemophilia B are notably expensive, costing $850,000 for both eyes, $2.1 million per injection, and over $3.5 million per injection, respectively. The high development costs coupled with the limited patient population for rare diseases contribute to the high prices of these treatments. To address these issues, preclinical trials in the United States are exploring the potential of modifying genes within the same viral vector to treat multiple diseases [115, 116]. Collaborative efforts between public and private sectors are crucial to advancing clinical development and enhancing efficiency.

5.3 HPP gene therapy

Despite the availability of ERT as an effective treatment for HPP, the discomfort associated with frequent injections and injection site reactions pose challenges for patients. Therefore, we aimed to employ gene therapy techniques to enable the patient's body to continuously and autonomously produce enzymes.

Intravenous therapy using AAV8 viral [117] and lentiviral vectors [118], fetal therapy using AAV9 viral vectors [119], bone marrow transplantation using lentiviral vectors [120], and intramuscular therapy using AAV8 viral vectors [121] have all demonstrated success in an HPP model, *Alpl*$^{-/-}$ mice. Bone marrow transplantation is challenging because of the risk of leukemia and burden of pretreatment on patients. Systemic administration of AAV is avoided to prevent systemic immune reactions and antibody production. Consequently, intramuscular injection is selected as the preferred method. AAV vectors offer safe and efficient gene delivery to nondividing cells

such as muscle cells, liver cells, and neurons, though the size limitation of inserted genes and the challenge of efficient production [103]. Moreover, AAV vectors exhibit tropism, allowing for the use of different subtypes tailored to specific organs [122]. Additionally, employing a tissue-specific promoter ensures that gene expression occurs exclusively within the targeted organ [122, 123]. Based on our previous study, intramuscular injection therapy using AAV8 viral vectors has emerged as the safest, most practical, and simplest approach.

5.4 Toward clinical application: AAV8-TANP-D10

Leveraging AAV organ tropism, we aimed to utilize AAV8, known for its tropism to infect liver and muscle tissues [122, 124], for localized injection into the muscle. The advantages of local administration include simplicity and safety, low risk of migration to other organs, suppression of immune responses associated with intravenous administration (i.e., antibody production and reactions that affect the whole body), and low potential for tumor formation. Furthermore, if adverse events cause the patient to discontinue enzyme expression, the muscle at the inoculation site can be surgically removed in the worst-case scenario.

AAV8-TNAP-D_{10}, a viral vector product expressing TNAP, is a gene therapy designed to extend lifespan, suppress symptom progression, and ameliorate symptoms in patients with HPP *via* a single intramuscular injection. AAV8-TNAP-D_{10} is an AAV8-TNAP vector with deca-aspartate (D_{10}) that targets the bone using a non-tissue-specific CAG promoter [121]. A single injection of AAV8-TNAP-D_{10} was administered into the thigh muscle of neonatal *Alpl*$^{-/-}$ mice (**Figure 3a**). Administration of AAV8-TNAP-D_{10} led to increased weight and a healthier appearance in *Alpl*$^{-/-}$ mice compared to untreated *Alpl*$^{-/-}$ mice (**Figure 3b**). The injection of AAV8-TNAP-D_{10} into the thigh muscle of neonatal *Alpl*$^{-/-}$ mice extended their lifespan and sustained elevated ALP activity in moribund *Alpl*$^{-/-}$ mice for 18 months at a dose of $\geq 3.0 \times 10^{11}$ vg/body. Micro-CT examination showed that bone maturation in treated *Alpl*$^{-/-}$ mice was comparable to that of wild-type mice (**Figure 3c**). Micro-CT examination of *Alpl*$^{-/-}$ mice's mandibles revealed that the mandibular structure in treated mice improved to the level of the wild-type mice. Moreover, there were notable improvements in the cementum and periodontal ligament structures compared to those in untreated mice. Enamel thickness, pulp structure volume, and thickness improved posttreatment to a degree where they could be assessed as comparable to those of the wild-type mice [125].

The use of *Alpl* $^{Prx1/Prx1}$ mice allows long-term studies of bones and teeth that are not possible with untreated lethal *Alpl*$^{-/-}$ mice [126]; moreover, they can be used as an adult HPP model or for tooth analysis. Treatment of *Alpl* $^{Prx1/Prx1}$ mice with AAV8-TNAP-D_{10} improves ossification, thus demonstrating that AAV8-TNAP-D_{10} is effective even in adult HPP models [127].

Prior to clinical trials, efficacy must be demonstrated in rodents and larger animals. A sheep model of HPP has been established in large animals using CRISPR-Cas9 technology to introduce a missense mutation (c.1077C > G; p.I359M), which serves as an effective model for studying alveolar bone conditions [128], but gene therapy has not yet been explored in that model. We selected the cynomolgus monkey (Macaca fascicularis) as a large animal model to assess the effectiveness of AAV8-TNAP-D_{10}. Our objectives were to 1) evaluate whether a single intramuscular injection of AAV8-TNAP-D_{10} maintained plasma ALP activity, 2) assess any potential antibody-mediated rejection of the protein expressed in animals with differing immune systems, and 3)

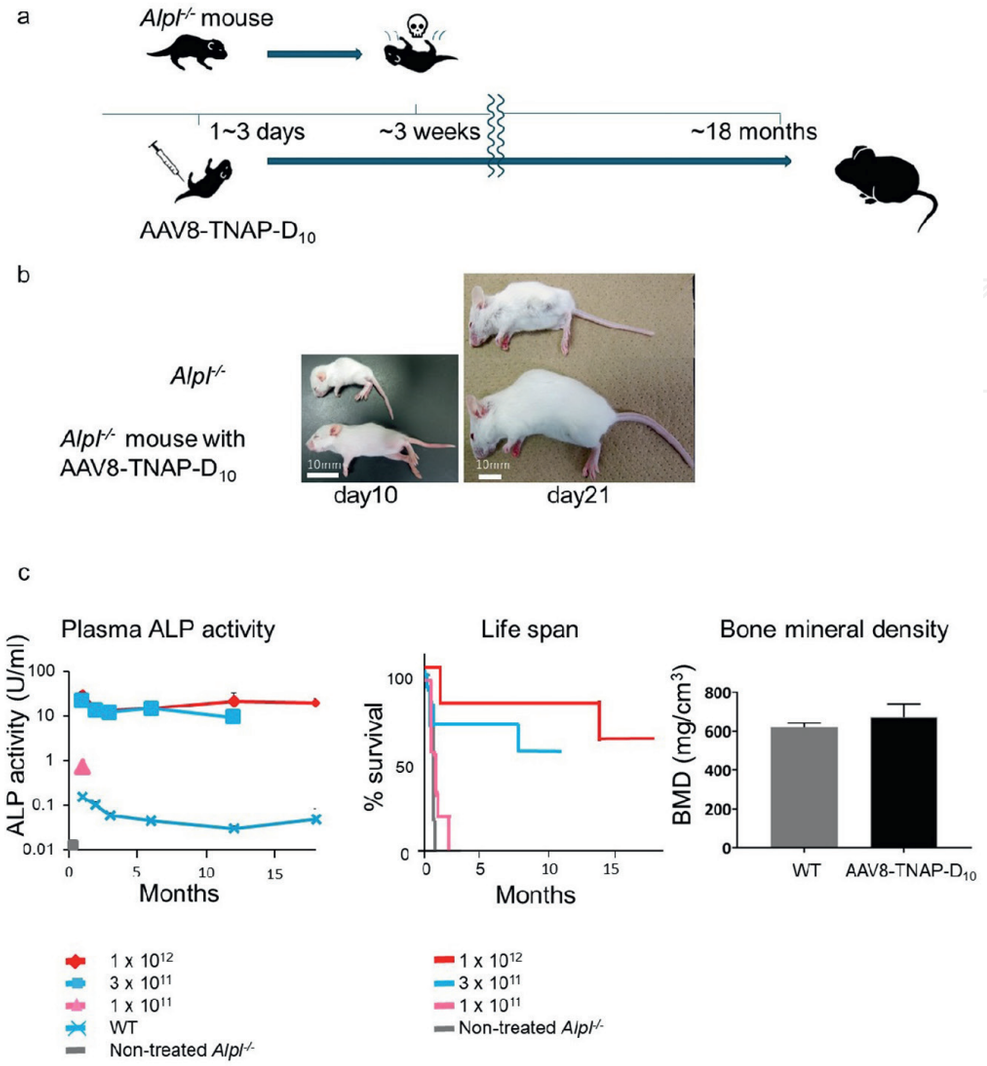

Figure 3.
Impact of one-time intramuscular AAV8-TNAP-D_{10} treatment on survival in $Alp^{-/-}$ mice. $Alpl^{-/-}$ mice injected with a single intramuscular injection of AAV8-TNAP-D_{10} in the quadriceps muscle within 3 days after birth ($1.0x10^{12}$ vg/body; n = 7, $3.0x10^{11}$ vg/body; n = 7, $1.0x10^{11}$ vg/body; n = 5) were observed for 18 months (a). Untreated model mice typically died before weaning. Physical appearance of untreated $Alpl^{-/-}$ mice vs. treated $Alpl^{-/-}$ mice (b). Plasma ALP activity, the survival curves, and bone mineral density at 18 months with computer tomography reconstruction of the femur bone. Bone mineral density at 18 months of age. CT reconstruction of the femur in AAV8-TNAP-D_{10} treated $Alpl^{-/-}$ mice ($1.0x10^{12}$ vg/body) vs. wild type (c).

examine adverse effects such as ectopic calcification, organ damage, or carcinogenesis, which have been previously reported following gene therapy failures.

AAV8-TNAP-D_{10} was injected into the lateral vastus lateralis muscle of two macaque monkeys under anesthesia. One monkey received 1×10^{13} vg/body and was observed for 266 days, whereas the other received 4×10^{13} vg/body (1×10^{13} vg at four sites) and was observed for 196 days. Both monkeys were compared to the controls. The rationale for increasing the number of the administration site instead of solely increasing the dosage was prevention of the leakage of the injected substance into the bloodstream from the muscle site, which could lead to inadvertent intravenous administration. The results showed that monkeys had higher serum ALP activity than the controls, which was maintained throughout the observation period. Although anti-TNAP antibodies appeared and caused a temporary decrease in serum ALP activity, these antibodies eventually decreased, thereby maintaining

high ALP activity. Anti-AAV antibodies also appeared but did not affect ALP activity. Regarding side effects, all blood tests, including those for liver function (aspartate aminotransferase, alanine aminotransferase), renal function (blood urea nitrogen, creatinine), and calcium metabolism (calcium, phosphate), showed no abnormalities. There were no pathological findings (no tumor formation or ectopic calcification), either macroscopically or microscopically. No local inflammation was observed at the injection site. Vector biodistribution was confirmed in each organ; furthermore, AAV8-TNAP-D_{10} was detected only in the skin and muscles, where it was administered (ongoing research and unpublished data).

6. Conclusions

We have discussed the treatment of HPP, the history of gene therapy, and our review of gene therapy for HPP. Although the approval of ERT has marked a revolutionary advancement, its reliance on regular and frequent injections significantly impacts the quality of life of patients and their families. Therefore, alternative treatments are urgently required.

In our study, AAV8-TNAP-D_{10} administered *via* a single intramuscular injection in both the early- and later-onset HPP mouse models demonstrated sustained elevation of serum ALP activity throughout the lifespan, along with improvements in bone and tooth ossification. No adverse effects were observed within this treatment range. In addition, AAV8-TNAP-D_{10} was shown to be safe and effective in primates. Thus, AAV8-TNAP-D_{10} intramuscular muscle injection treatment appears to have promising efficacy and safety profile for clinical application.

Given the costs associated with gene therapy as well as the broad spectrum of symptoms in patients with HPP, it is crucial to address societal considerations regarding patient selection for gene therapy.

Our future studies will include safety assessments and further investigations in preparation for clinical trials. We intend to advance our research efforts in the pursuit of enhanced safety profiles.

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Conflict of interest

TM and KM have previously received research funding from Aruvant Sciences and held an endowed chair affiliated with Aruvant Sciences during the course of the research. JLM received partial research funding from Aruvant Sciences.

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