



Full Length Article

Recovery of bone mineralization and quality during asfotase alfa treatment in an adult patient with infantile-onset hypophosphatasia



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ABSTRACT

Hypophosphatasia (HPP) is a hereditary musculoskeletal disorder characterized by low serum alkaline phosphatase (ALP) activity leading to poor bone mineralization. On a micro-morphological level, this may not only be reflected by an enrichment of osteoid but also a degradation of bone quality. Asfotase alfa is an enzyme replacement therapy that was recently demonstrated to improve bone mineralization as well as clinical status (e.g. growth, muscle strength and quality of life). However, the underlying changes of bone quality parameters on asfotase alfa treatment are currently not known. In the present study, we report a 24-year-old woman with genetically confirmed infantile-onset HPP and recurrent fractures. While the initiated asfotase alfa treatment was followed by rapid clinical improvements (i.e., disappearance of bone marrow edema, increase of muscle strength), the BMD assessed by DXA at the hip and spine increased moderately at two years follow-up. A detailed skeletal assessment using high-resolution peripheral quantitative computed tomography (HR-pQCT) and a high-resolution analysis of two consecutive iliac crest bone biopsies revealed only minor improvements of bone microarchitecture but a remarkable reduction of osteoid parameters. Furthermore, the high mineralization heterogeneity at baseline assessed by quantitative backscattered electron imaging (qBEI) decreased after 2 year of asfotase alfa treatment. Finally, we found an increase in mineral maturation reflected by higher mineral-to-matrix and carbonate-to-phosphate ratios using Fourier transform infrared spectroscopy (FTIR) imaging as well as increased local mechanical properties using reference point indentation (RPI). Taken together, our findings provide evidence for an improvement of bone quality indices beyond the mere reduction of osteoid indices and thereby contribute to the understanding of fracture risk reduction in HPP patients on asfotase alfa treatment.

1. Introduction

Hypophosphatasia (HPP) is a rare musculoskeletal disorder characterized by genetic defects of the alkaline phosphatase gene *ALPL* encoding the tissue non-specific alkaline phosphatase (TNSALP) and resulting in low serum alkaline phosphatase (ALP) activity and subsequent accumulation of pyridoxal-5-phosphate (PLP) and other TNSALP substrates such as inorganic pyrophosphate (PPI) [1]. PPI is a potent inhibitor of bone mineralization causing an enrichment of non-mineralized bone matrix (osteoid) [2]. Clinically, the reduced bone mineralization leads to an increased risk of fractures and fracture healing complications [3]. While the perinatal and infantile-onset forms can be associated with severe and potentially life-threatening symptoms, HPP in adults commonly presents with moderate symptoms such

as early loss of permanent teeth, musculoskeletal pain and stress fractures [4].

Asfotase alfa (Alexion Pharmaceuticals, Inc., Boston, MA, USA) is an enzyme replacement therapy (ERT) that is approved for the treatment of pediatric-onset HPP. It was shown to improve survival for perinatal and infantile HPP [5]. Furthermore, skeletal mineralization defects in children significantly improved only months after the initiation of the treatment [6]. In adults and adolescents with pediatric-onset HPP, there are only few reports that have described the treatment response to asfotase alfa. Site-specific improvements of bone microarchitecture assessed by HR-pQCT [7] and significant promotion of fracture healing [8] were demonstrated. Furthermore, positive five-year efficacy and safety was recently demonstrated in a cohort of 19 adult patients with HPP [9].

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We have previously demonstrated that bone biopsies of adult patients with HPP are characterized by an enrichment of osteoid accompanied by a degradation of bone quality parameters such as decreased calcium content (CaMean) assessed by quantitative backscattered electron imaging (qBEI) [10]. While the diminished bone quality might contribute to the increased fracture risk of the affected individuals, the changes of these bone matrix mineralization parameters have not been described in adult HPP patients receiving asfotase alfa treatment. Furthermore, other compositional aspects of bone biopsies from HPP patients (both treatment-naïve and follow-up) are currently not known. Therefore, the aim of the present study was to determine these detailed clinical and compositional properties in an adult patient with pediatric-onset HPP and to demonstrate their changes in response to enzyme replacement therapy with asfotase alfa. We specifically investigated the bone microarchitecture, osteoid parameters, osteocyte lacunar density, bone mineral density distribution (BMDD), Fourier transform infrared spectroscopy (FTIR) parameters and local mechanical properties (reference point indentation, RPI).

2. Material and methods

2.1. Patient studies

A 24-year old female presented at our department with severe pain in the left knee. She had been diagnosed with infantile-onset HPP. Her symptoms included muscle weakness, respiratory complications and early loss of deciduous teeth. The patient developed severe persistent muscle pain and muscle weakness during adolescence. She also complained about repetitive headache. Her current height was 157 cm with a weight of 50.6 kg. During adolescence she suffered from a right tibial shaft fracture treated with an intramedullary nail, which was followed by delayed fracture healing. As the patient currently suffered from persistent pain in the left knee (pain level 7/10 on the visual analogue scale, VAS), an MRI was conducted and showed a stress fracture with bone marrow edema in the tibia plateau. Additional CT scans pointed to hole-like structures in the area of the bone marrow edema. The patient also suffered from pain in the right foot, and another bone marrow edema in the talus was observed. In the course of asfotase alfa treatment, she sustained a non-displaced distal radius fissure fracture from a trauma of unclear energy (fall while dancing) which healed regularly.

Asfotase alfa treatment was initiated at a dosage of 1 mg/kg subcutaneous injection six times per week. The treatment was well tolerated apart from a moderate injection site reaction that resolved completely after the first two weeks. No signs of ectopic calcifications were observed in carotid, abdominal and renal ultrasound examinations at 3, 12 and 24 months after treatment initiation.

This study reports the detailed data of a study period of the first two years of treatment. Informed written consent was obtained from the patient. We regularly assessed laboratory values including alkaline phosphatase (ALP) activity and pyridoxal-5-phosphate levels. Furthermore, muscle strength was quantified at baseline and after 8 and 16 months by hand grip strength measurements and chair rising test combined with a ground force reaction plate (Leonardo GFRP, Novotec Medical GmbH, Pforzheim, Germany) as described previously [11]. The areal bone mineral density (aBMD) was evaluated using Dual Energy X-ray absorptiometry (DXA, Lunar iDXA, GE Healthcare; Madison, WI, USA) at baseline and after 2 years. High-resolution peripheral quantitative computed tomography (HR-pQCT; XtremeCT, Scanco Medical, Switzerland) was performed for initial diagnosis and after two years of treatment at the non-dominant distal radius and tibia in a standardized procedure as previously described by our group [12]. Volumetric bone mineral density (vBMD) measurements included trabecular BMD (Tb.BMD, mgHA/cm³) and cortical BMD (Ct.BMD, mgHA/cm³), while bone microstructure values are similar to those used in bone histology and include bone volume per tissue volume ratio (BV/TV), trabecular number (Tb.N, 1/mm) and thickness (Tb.Th, mm) as well as cortical

thickness (Ct.Th, mm).

2.2. Histology and histomorphometry

In order to further evaluate treatment efficacy, two diagnostic transiliac crest biopsies were obtained according to Bordier [13] at the time of asfotase alfa treatment initiation and after two years. The specimens were fixed in 3.7% formaldehyde, dehydrated, embedded in methyl-methacrylate, and cut on a Microtec rotation microtome (CVT 4060E, Micro Tec, Walldorf, Germany). Afterwards the 5- μ m sections were stained by toluidine blue, trichrome Masson-Goldner and von Kossa. Histomorphometric analysis was performed according to the ASBMR nomenclature committee [14]. The following parameters for bone microstructure and osteoid quantification were evaluated in the von Kossa stained sections: BV/TV, Tb.N, Tb.Th, Tb.Sp; osteoid volume per bone volume (OV/BV), osteoid surface per bone surface (OS/BS) and osteoid thickness (O.Th). An area of approximately 2 mm² was evaluated in each of the sections.

2.3. Quantitative backscattered electron imaging (qBEI)

QBEI was performed using a scanning electron microscope (LEO 435 VP; LEO Electron Microscopy Ltd., Cambridge, England) with a backscattered electron detector (Type 202; K.E. Developments Ltd., Cambridge, England) as described previously [15,16]. The scanning electron microscope was operated at 20 kV and 680 pA at a constant working distance. A total area of approximately 1 mm² per specimen was analyzed. The acquired images were analyzed using a customized MATLAB (TheMathWorks, Inc. Natick, Massachusetts, USA) script. QBEI was used to measure the bone mineral density distribution based on the generated grey values that represent the mean calcium content (mean Ca-Wt%). For each sample, the mean calcium content (CaMean), mineralization heterogeneity (CaWidth) and the most frequent calcium content (CaPeak) were evaluated. Moreover, the number of osteocyte lacunae per bone area (N.Ot.Lc/B.Ar.) and the mean osteocyte lacunar area (Ot.Lc.Ar) was calculated from the images. The results of the two biopsies were compared to a healthy, age- and sex-matched control (female, 24 years) from our archive previously collected in the context of another study [17].

2.4. Fourier transform infrared spectroscopy (FTIR)

FTIR was carried out for complementary bone compositional analysis in terms of mineral and matrix characterization. 2 μ m thin sections were cut with a microtome and subsequently placed on barium fluoride (BaF₂) windows. Background scans were performed on the BaF₂ window. Specimens were scanned at the trabecular region in transmission mode on a Spotlight 400 system (Perkin Elmer, Waltham, MA, USA) attached to a Frontier spectroscope (Perkin Elmer, Waltham, MA, USA) with a pixel size of 6.25 μ m. Spectra were acquired over a spectral range of 4000 to 570 cm⁻¹. Each pixel was scanned 12 times, automatically averaged for better signal to noise ratio and background subtracted using the software of the manufacturer (SpectrumIMAGE R 1.7.1.0401 - Perkin Elmer, Waltham, MA, USA). Further data processing was carried out using a customized MATLAB (TheMathWorks, Inc. Natick, Massachusetts, USA) routine. Mineral-to-matrix ratio (MMR) was calculated by dividing the area of the phosphate peak (1154–900 cm⁻¹) by the area of the amide I peak (1700–1600 cm⁻¹). Carbonate-to-phosphate ratio (CPR) was determined by dividing the area of the peak at 890–850 cm⁻¹ and 1154–900 cm⁻¹, respectively.

2.5. Reference point indentation (RPI)

RPI was carried out on embedded bone samples. The cortical area of the bone was used for indentation. Coplanar polished samples were mounted to an X-Y-table for controlling the position of each indent and

to control the distance between two neighboring indents. For indentation a BioDent h_{fc} Reference Point Indentation instrument (Active Life Scientific Inc., Santa Barbara, CA, USA) was used, equipped with a BP2 probe (Active Life Scientific Inc., Santa Barbara, CA, USA). Ten indentations per cortical bone region were performed while maintaining a sufficient distance in between the individual indentations. Individual indentations were performed at a force of 6 N at 2 Hz for 10 indentation cycles. Load-displacement curves were recorded using Biodent Software (Active Life Scientific Inc., Santa Barbara, CA, USA, Supplementary Fig. 1). Subsequently, each indent was double-checked with opto-digital microscopy to rule out that indentations were placed in voids or soft tissue. Indents that were partially placed in canals or cracks were excluded from the statistics. RPI indices as 1st Cycle Indentation Distance (ID 1st - μm), Total Indentation Distance (TID - μm), Indentation Distance Increase (IDI 1st - μm) and Average Energy Dissipated (Avg ED - μJ) were calculated.

3. Results

At the time of the initial presentation at our clinic, a laboratory assessment showed an AP activity of 9 U/l (reference range 35–104 U/l) and pyridoxal-5-phosphate levels of 162.5 $\mu\text{g/l}$ (reference range 7.5–18.5 $\mu\text{g/l}$). Genetic testing revealed the heterozygous ALPL mutation c.1001G > C (P.G334D). This mutation was previously described to cause HPP with a dominant negative effect and associated with a strongly reduced residual TNSALP activity [18].

After 8 weeks of asfotase alfa treatment, the pain in the left knee was strongly reduced (VAS 1/10) and a new MRI demonstrated almost complete disappearance of bone marrow edema (Fig. 1A). While AP levels increased directly after the start of treatment, elevated PLP levels decreased to reference range (Fig. 1 B, C) and remained in the reference range for the rest of the treatment time. The muscle pain of the patient resolved after several weeks (from VAS 6/10 to 2/10), and the muscle function measured by chair rising test and grip strength test improved after 8 and 16 months (Fig. 1D, E). The patient was regularly checked by ultrasound of the vessels and kidneys but no signs of ectopic mineralization were detected.

In order to determine the treatment response, we reassessed the patient using DXA and HR-pQCT as well as follow-up iliac crest biopsy. DXA BMD improved both at the left hip (+3.9%) and lumbar spine (+4.2%). (Table 1). In the HR-pQCT analysis, cortical and trabecular volumetric BMD as well as overall bone microarchitecture remained constant. At the distal tibia, a notable decrease of trabecular number was found, while an increase of trabecular thickness was detected.

The histomorphometric analysis of the initial iliac crest biopsy revealed increased osteoid parameters (volume, surface and thickness) in the range of osteomalacia. Moreover, irregular mineralization fronts were noted (Fig. 2). After two years of treatment, a slight reduction of BV/TV along with a decrease of Tb.N but an increase of Tb.Th could be noted. Importantly, osteoid volume decreased by 74%, which was due to both a decrease of osteoid thickness and osteoid surface (Fig. 2, Table 2).

qBEI was conducted to demonstrate the changes of bone matrix mineralization in response to asfotase alfa treatment (Fig. 3 A). Asfotase alfa led to a small increase in the mean mineralization (CaMean) from 23.51 to 23.58 wt% (Fig. 3 B), while the CaMean of both HPP biopsies was only slightly reduced compared to the control (23.99 wt%). Interestingly, the mineralization heterogeneity (CaWidth) decreased by 17.2% between baseline and 2 years and reached almost the values of the healthy control after two years of treatment (Fig. 3 C). Bone mineral density distribution (BMDD) curves demonstrate how the matrix mineralization changed after treatment towards a profile associated with healthy bone tissue (Fig. 3 D). The number of osteocyte lacunae per bone area remained equal, the lacunar area decreased from 33.87 to 32.77 μm^2 (Table 3).

Additionally, we conducted an FTIR analysis to obtain further

information on the changes of mineralization after asfotase alfa treatment. The mineral-to-matrix ratio increased by 11% after two years of treatment indicating higher mineralization (Fig. 4 A). The carbonate-to-phosphate ratio also increased by 5.8% indicating either increase of carbonate or decrease of phosphate content (Fig. 4 B).

We conducted RPI analysis to gain information about the local mechanical behavior before and after asfotase alfa treatment as well as in a control sample (Table 4). Here, ID 1st decreased by 12% after therapy when compared to baseline. TID decreased by the same order of magnitude. IDI decreased by 18% following asfotase alfa treatment. Avg ED showed an increase of 15.3% following therapy.

4. Discussion

Although HPP is known to cause impaired bone mineralization, detailed bone quality characteristics and especially their changes on asfotase alfa treatment have not been reported so far. Clinically, asfotase alfa treatment in adult and adolescent HPP patients was found to lead to improved skeletal radiographic findings [19], enhanced fracture healing [8] and muscle pain reduction [9,20]. The current study aimed to investigate the underlying structural and compositional changes of bone in an adult HPP patient with improved fracture healing and muscle function on asfotase alfa treatment. More specifically, we applied various analysis methods (i.e., HR-pQCT, undecalcified histology, qBEI and FTIR) to determine the bone density, microarchitecture, mineralization, osteocyte lacunar dimensions and mineral characteristics before and after treatment.

Of note, asfotase alfa treatment resulted in a moderate increase of BMD assessed by DXA, but no major improvements of bone microstructure assessed by HR-pQCT. A previous case study demonstrated an unchanged bone microstructure at the distal radius and an increase of both trabecular and cortical parameters at the distal tibia in a 36-year-old male patient after 12 months of treatment [7]. We also did not find any microstructural changes at the distal radius, however a simultaneous decrease of trabecular number and increase of trabecular thickness was detected in our patient at the distal tibia. Since osteoid is not detected by HR-pQCT, the increase in trabecular thickness may be due to remineralization of parts of the osteoid; however, these assumptions could only be confirmed by other techniques involving undecalcified histology.

While the histomorphometric analysis confirmed only minor changes of bone microstructure, the detected osteoid accumulation found in the initial biopsy is a hallmark of the disease [10]. We here demonstrated a strong reduction of osteoid indices following asfotase alfa treatment, thereby underlining the importance of bone biopsies to assess the efficacy of this treatment. Matching our findings, a recent study in 19 adult HPP patients found that mean osteoid indices also decreased [9]. Next to the partial correction of hyperosteoridosis, we detected an amelioration of mineralization heterogeneity and other compositional parameters. For instance, BMDD showed a trend towards recovery following asfotase alfa treatment reaching almost the values of the healthy control.

As fractures and fracture healing complication strongly affect the quality of life in HPP patients, the prevention of further fractures is a major aim in the treatment of HPP patients. Indeed, asfotase alfa treatment has been reported to decrease fracture risk in HPP patients of different ages [5,9]. In this context it is important to mention that increased fracture risk has been associated with osteoid accumulation and/or increased mineralization heterogeneity in patients with osteoporosis [21], femoral neck fractures [22] and fragility fractures [23], as well as in pediatric patients [24] and patients with chronic kidney disease [25]. Similarly, bone tissue covered by excessive osteoid seams showed signs of premature aging in cases afflicted with vitamin D-deficient osteomalacia and reduced fracture resistance [26]. In the current study, we observed strong reductions of osteoid accumulation and mineralization heterogeneity in the histological and qBEI analyses after

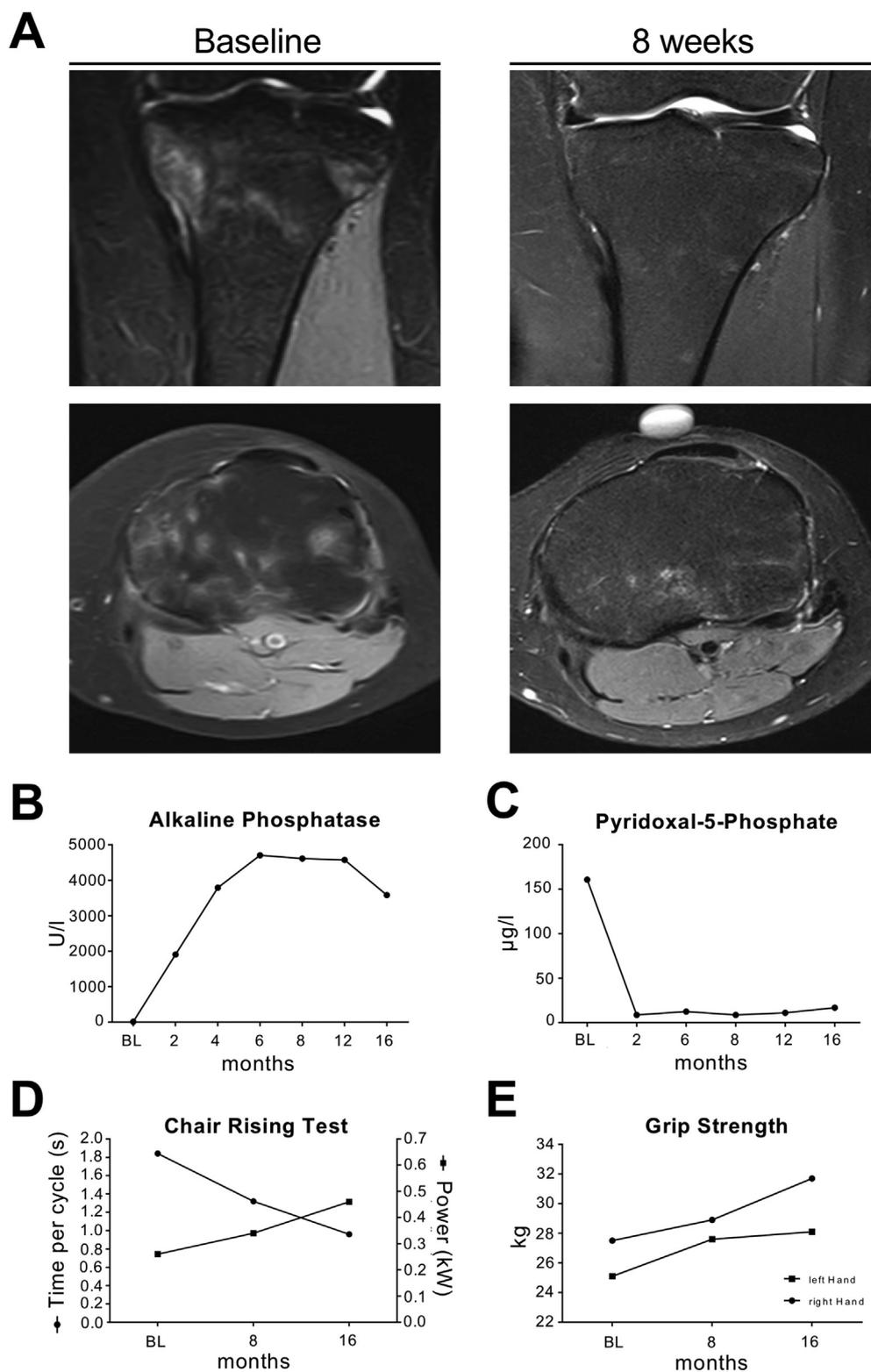


Fig. 1. Clinical improvements after asfotase alfa treatment in a 24-year-old adult HPP patient. (A) MRI scans (coronal view, proton-density weighted sequence) at baseline and after 8 weeks of treatment show a bone marrow edema/stress fracture in the proximal tibia that fracture healed regularly. (B) AP levels increased rapidly after treatment initiation and remained elevated. (C) PLP levels decreased and remained within the reference range throughout the treatment period. (D) Chair rising test combined with a ground force reaction plate indicated a continuous decrease of time per cycle (s) and a continuous increase in maximum power (kW) after 8 and 16 months of treatment. (E) Grip strength similarly increased in both hands at 8 and 16 months.

two years of asfotase alfa treatment. Therefore, these changes in bone matrix mineralization may be the major factor responsible for the reduction of fracture risk in HPP patients under asfotase alfa treatment.

Next to the changes in matrix mineralization between baseline and two years of treatment, we observed no major changes in osteocyte lacunar number and size. However, a slight reduction of osteocyte lacunar area was detected. This may reflect the improvement of bone mineralization with regard to the notion that an enlargement of

osteocytes has been detected in conditions of calcium deficiency such as lactation [27] and vitamin D deficiency [26,28]. Using FTIR imaging, we further quantified the changes of tissue mineralization (i.e., mineral-to-matrix and carbonate-to-phosphate ratio). The increase of mineral-to-matrix ratio complemented the qBEI results in terms mineralization recovery. At the same time, we observed an increase in the carbonate-to-phosphate ratio. Higher carbonate-to-phosphate ratio reflects either increased carbonate or decreased phosphate content in response to

Table 1

DXA and HR-pQCT values at baseline and after 2 years of asfotase alfa treatment.

DXA	Left hip		Spine (L1–L2)	
	Baseline	2 ys.	Baseline	2 ys.
BMD (g/cm ²)	0.843	0.876	0.948	0.988
T-Score	-1.3	-1	-1.8	-1.5
Z-Score	-1	-0.6	-1.3	-0.9

HR-pQCT	Radius		Tibia	
	Baseline	2 ys.	Baseline	2 ys.
Tb. BMD (mg HA/cm ³)	137.4	133.7	182.1	167.4
Ct. BMD (mg HA/cm ³)	816.8	818.4	819.6	830.6
BV/TV (%)	11.5	11.1	15.2	14
Tb.N (1/mm)	1.75	1.78	2.02	1.52
Tb.Th (mm)	0.063	0.062	0.075	0.092
Ct.Th (mm)	0.71	0.7	0.92	0.9

Table 2

Histomorphometry results demonstrating the reduction of osteoid parameters. Bold indicates abnormal values compared to reference values (i.e. OV/BV < 5%, OS/BS < 20% and O-Th < 12 μm [17]).

Histology	Baseline	2 ys.
BV/TV (%)	17.6	15.2
Tb.Th (μm)	123.5	141.4
Tb.N (1/mm)	1.43	1.07
Tb.Sp (μm)	577.6	790.4
OV/BV (%)	12.4	3.2
OS/BS (%)	66.4	34.1
O-Th (μm)	12.95	7.31

asfotase alfa treatment and was previously linked to increased bone maturity [29], carbonate substitution by hydroxyapatite [30] and increased crystallinity [31]. Low carbonate-to-phosphate ratios were detected in patients with hyperosteoridosis due to renal osteodystrophy [30].

Utilizing RPI, we found local bone mechanical property changes following treatment. The degree of matrix mineralization often scales with the depth of indentation. A trend towards physiological bone hardness values was found after treatment as indicated by the

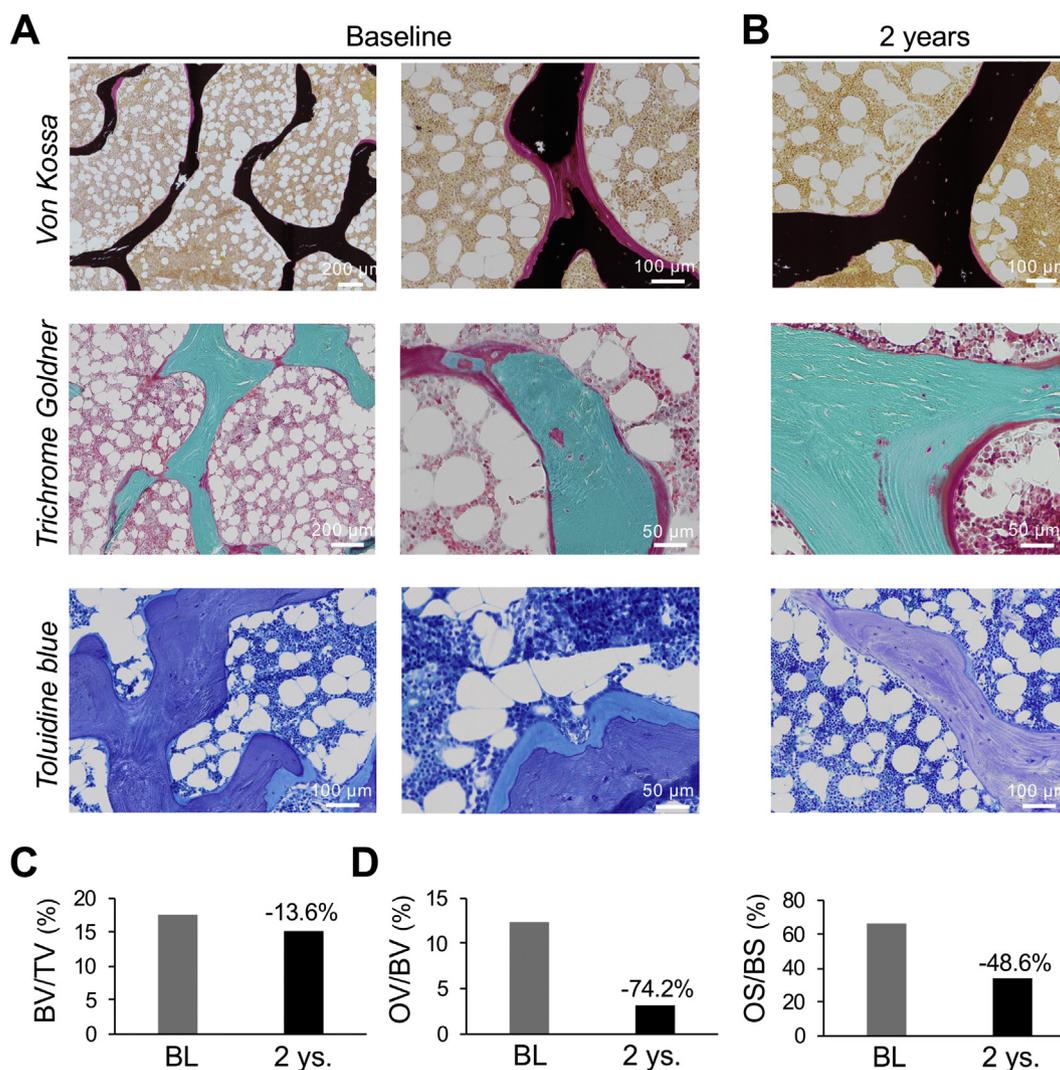


Fig. 2. Histological changes in undecalcified histology. (A) At baseline, a detailed histological assessment revealed osteoid accumulation with osteoid “bridging” the mineralized trabeculae. (B) After 2 years of treatment, a slight decrease of BV/TV but a substantial decrease of osteoid parameters was noted. (C) Bone volume per tissue volume (BV/TV). (D) Osteoid volume per bone volume (OV/BV). (E) Osteoid surface per bone surface (OS/BS). More detailed histomorphometric data is presented in Table 2.

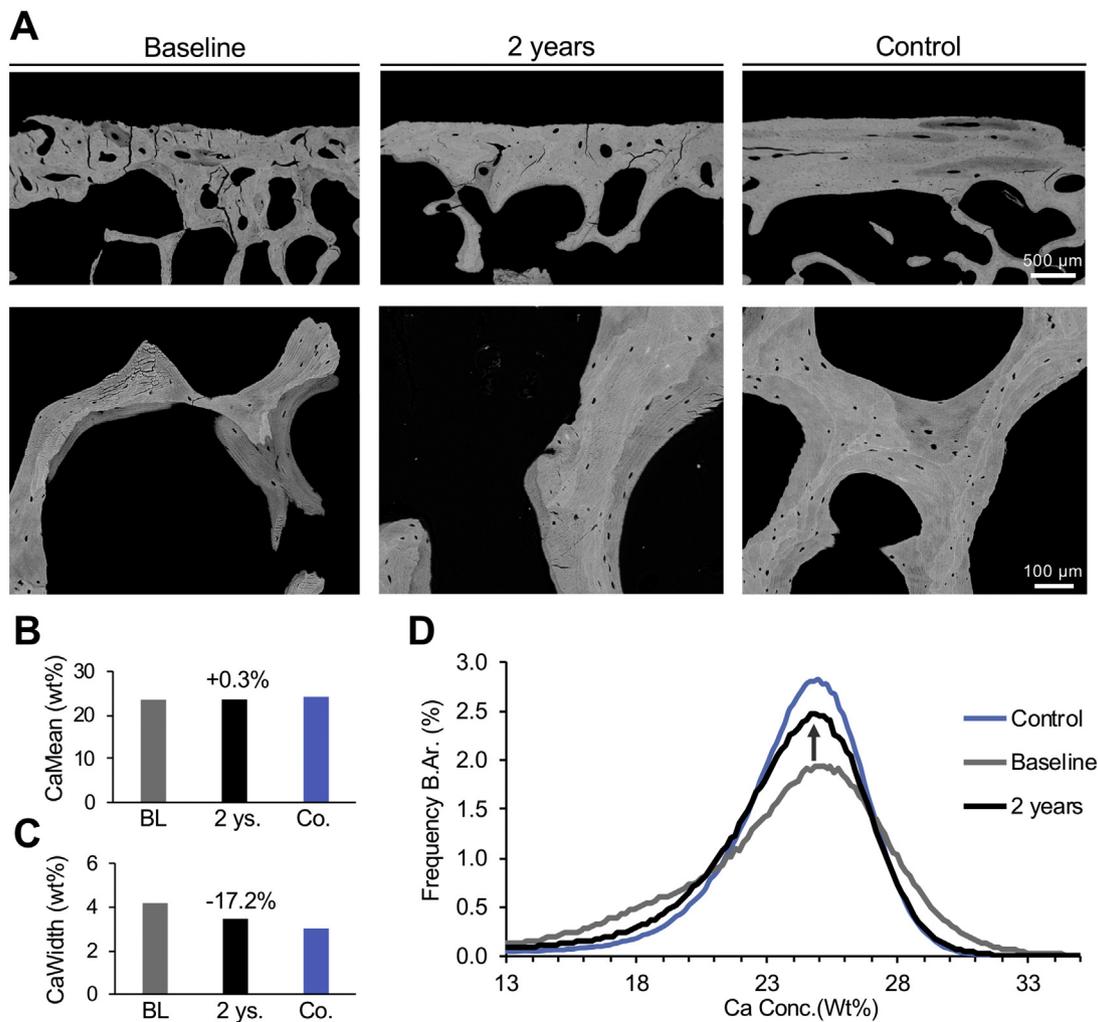


Fig. 3. Decrease of mineralization heterogeneity after treatment. (A) Exemplary qBEI images showing low mineralized bone matrix (dark areas) in HPP before treatment as well as the improvement of bone mineralization after treatment and compared to an age-matched female control. (B) CaMean was only slightly lower in HPP (23.51 wt%) compared to the control (23.99 wt%) and remained almost equal after treatment (23.58 wt%). (C) Mineralization heterogeneity (CaWidth) was higher in HPP (4.18 wt%) compared to the control (3.46 wt%) and decreased after treatment reaching almost the value found in the control (2.97 wt%). (D) Histograms indicate that normalization of the bone mineral density distribution (BMDD) occurs during treatment. More detailed qBEI data is presented in Table 3.

Table 3
Quantitative backscattered electron imaging (qBEI) results at baseline and after 2 years of asfotase alfa treatment.

Parameter	Baseline	2 ys.	Control
CaMean (wt-%)	23.51	23.58	23.99
CaPeak (wt-%)	25.12	24.64	24.96
CaWidth (wt-%)	4.18	3.46	2.97
N.Ot.Lc/B.Ar (1/mm ²)	234.67	232.74	n/a
Ot.Lc.Ar (μm ²)	33.87	32.77	n/a

decreased ID 1st. In addition, higher energy dissipating capabilities were found in treated bone tissue, which is likely undergoing mineralization processes due to treatment and thus points to a recovery of physiological fracture resistance.

The limitations of our study include the fact that only one HPP patient on treatment could be analyzed in detail, which hinders possible statements on statistical significance. Nonetheless, the collection of structural and compositional data contributes to the understanding of the effects of enzyme replacement therapy with asfotase alfa. Comparing the baseline parameters with a follow-up biopsy and a healthy control, we found a partial recovery of most parameters. Further improvements are likely considering that complete skeletal

remodeling takes longer than the current study period of 2 years. Furthermore, the observation that treatment discontinuation has been linked to reappearance of unmineralized bone on a radiographic level [32] points to the necessity of a lifelong treatment.

In conclusion, this is the first study to assess the bone quality in an adult HPP patient before and after asfotase alfa treatment. Next to the clinical improvements in terms of stress fracture healing and increase of muscle strength, asfotase alfa treatment led to a recovery of bone mineral density distribution assessed by qBEI, remarkable reduction of osteoid as well as increases in the mineral-to-matrix and carbonate-to-phosphate ratio assessed by FTIR. It is now required to analyze the changes of bone quality characteristics in larger HPP cohorts on asfotase alfa treatment with special regard to fracture risk.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bone.2019.05.036>.

Disclosures

FB received honoraria as a speaker and consultant from Alexion Pharmaceuticals, Inc.

TR, TS, FNS, SVK, BB and MA declare that they have no conflict of interest.

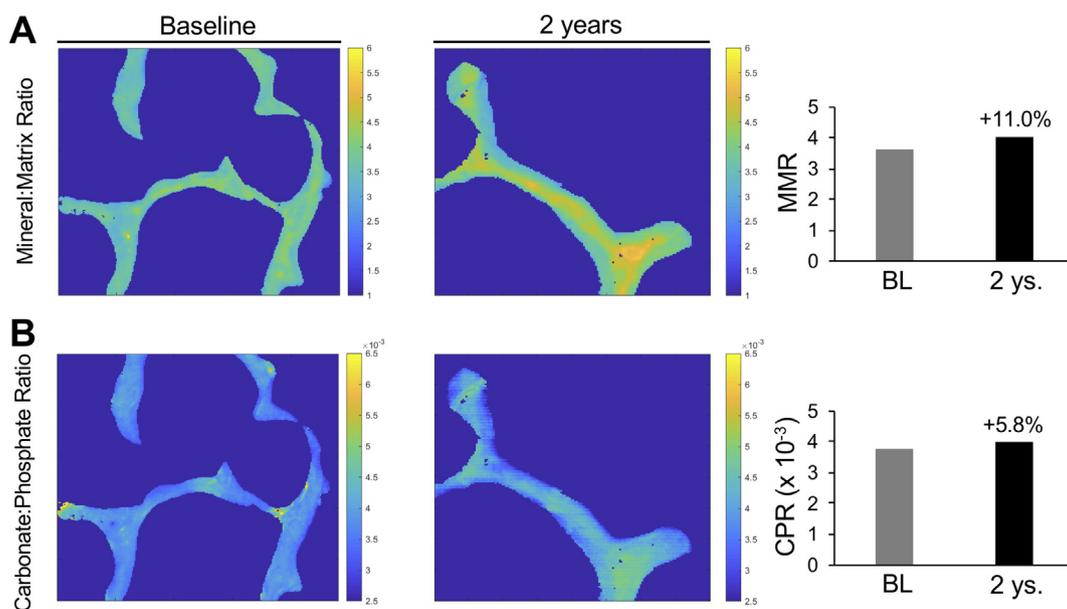


Fig. 4. Changes of mineral characteristics assessed by Fourier transform infrared (FTIR) spectroscopy. FTIR maps indicate that (A) Mineral-to-matrix ratio (MMR) increased by 11% and (B) carbonate-to-phosphate ratio (CPR) increased by 5.8% after two years of asfotase alfa treatment.

Table 4

Reference Point Indentation (RPI) of mineralized hard tissue.

Parameter	Baseline	2 ys.	Control
ID 1st (µm)	56.56	49.80	51.54
TID (µm)	60.05	52.88	54.65
IDI 1st (µm)	7.36	6.02	5.92
Avg ED (µJ)	14.25	16.44	15.66

ID 1st, 1st Cycle Indentation Distance; TID, Total Indentation Distance; IDI 1st, Indentation Distance Increase; Avg ED, Average Energy Dissipated.

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