JBMR Plus, 2024, **8**, ziae107
https://doi.org/10.1093/jbmrpl/ziae107
Advance access publication: August 10, 2024
Case Study

Longitudinal course of circulating miRNAs in a patient with hypophosphatasia and asfotase alfa treatment: a case report

Benjamin Hadzimuratovic[1,2], Judith Haschka[1,2], Matthias Hackl[3], Andreas B. Diendorfer[3], Andreas Mittelbach[4], Julia Feurstein[1,2], Jochen Zwerina[1,2], Heinrich Resch[5,6], Roland Kocijan[1,2,6,*]

[1]1st Medical Department, Ludwig Boltzmann Institute of Osteology at Hanusch Hospital of OEGK and AUVA Trauma Centre Meidling, Hanusch Hospital, 1140, Vienna, Austria
[2]1st Medical Department, Hanusch Hospital, 1140, Vienna, Austria
[3]TAmiRNA GmbH, 1110, Vienna, Austria
[4]Institute of Physical Medicine at Hanusch Hospital, 1140, Vienna, Austria
[5]2nd Department of Internal Medicine - VINFORCE, St. Vincent Hospital, 1060, Vienna, Austria
[6]Medical Faculty of Bone Diseases, Sigmund Freud University, 1020, Vienna, Austria

*Corresponding author: Roland Kocijan, MD, Ludwig Boltzmann Institute of Osteology, Heinrich-Collin-Straße 30, 1140, Vienna, Austria (roland.kocijan@osteologie.lbg.ac.at)

Abstract

Hypophosphatasia (HPP) is characterized by low activity of tissue nonspecific alkaline phosphatase (TNSALP). The enzyme replacement therapy asfotase alfa has been approved for childhood-onset forms of HPP. MicroRNAs (miRNAs) have emerged as a novel disease biomarker, with potential application in therapy monitoring. Circulating miRNAs were analyzed at baseline, months 1, 2, 4, and 16 in a 49-yr-old woman with childhood-onset HPP, chronic musculoskeletal pain, and non-traumatic fractures prior to enzyme replacement therapy. Serum RNA was extracted and sequenced using miRNeasy Mini Kit (Qiagen, Germany), RealSeq Biosciences Kit (Santa Cruz, US) together with miND spike-in control kit (TAmiRNA, Austria) and Illumina NovaSeq 6000 SP1 flow cell (San Diego, US). Brief Pain Inventory Severity and Interference scores (BPI-S/BPI-I), fatigue severity scale (FSS), Patient Global Impression of Improvement (PGI-I), Western Ontario and McMaster university hip disability and osteoarthritis outcome score (WOMAC), fibromyalgia impact questionnaire (FIQ), 6-Minute Walking Test (6-MWT), chair-rise-test (CRT), and handgrip dynamometry (HD) were performed at baseline and different timepoints during the therapy. Out of >800 screened, 84 miRNAs were selected based on differences in expression profiles between 24 HPP patients and 24 healthy controls. Six miRNAs showed a clear graphic trend and were up- or downregulated by ≥50% reads per million (rpm). These included hsa-let-7i-5p (+50%), hsa-miR-1-3p (−66.66%), hsa-miR-1294 (+63.63%), hsa-miR-206 (−85.57%), hsa-miR-375-3p (−71.43%), and hsa-miR-624-5p (+69.44%). hsa-miR-1-3p and hsa-miR-206 were identified as muscle-specific miRNAs. hsa-mir-375-3p, which negatively regulates osteogenesis, was significantly downregulated. In terms of patient-reported outcomes, BPI-S, BPI-I, FSS, PGI-I, WOMAC, and FIQ showed a reduction by −58.62%, −68.29%, −33.33%, −75.00%, −63.29%, and −43.02%, respectively. 6-MWT improved by +33.89% and CRT by −44.46%. Mean hand grip strength of the right/left hand measured by HD improved by +12.50% and + 23.53%, respectively. miRNA profile changes during the therapy with asfotase alfa, accompanying improvements in functionality tests and quality of life scores.

Keywords: hypophosphatasia, HPP, micro-RNAs, miRNAs, ALP, asfotase alfa, enzyme replacement therapy, ERT

Lay Summary

Hypophosphatasia (HPP) is a rare genetic bone disease characterized by low activity of an enzyme involved in mineralization of bony tissues, the nonspecific alkaline phosphatase (TNSALP). The enzyme replacement therapy with recombinant asfotase alfa has been approved recently. MicroRNAs (miRNAs) are small signaling molecules, which are involved in the regulation of gene expression and signaling pathways. They emerged as a novel disease biomarker, with potential application in therapy monitoring. We analyzed the levels of miRNAs of a 49-yr-old woman with childhood-onset HPP during the therapy. The patient suffered from musculoskeletal pain and non-traumatic fractures before the begin of enzyme replacement therapy. miRNAs in blood and clinical tests were performed at baseline and different timepoints. Blood levels of 6 miRNAs showed a clear graphic trend and were elevated or lowered by ≥50% units (reads per million) during the therapy. Patient-reported questionnaires showed a reduction of at least a third, reflecting clinical improvement of symptoms during the therapy. The scores of 6-MWT and CRT and hand grip also improved, signaling improvements in muscle functionality under asfotase alfa. miRNA profile changes during the therapy with asfotase alfa, accompanying improvements in functionality tests and quality of life scores.

Received: May 8, 2024. **Revised:** July 20, 2024. **Accepted:** July 25, 2024

Introduction

Hypophosphatasia (HPP) is a rare genetic disorder of metabolism caused by more than 400 different loss-of-function mutations in the tissue-nonspecific alkaline phosphatase (TNSALP) gene.[1] HPP is highly variable in clinical expression and is generally classified into 6 forms based on the age of onset and the clinical features: perinatal severe, perinatal benign, infantile, childhood, adult, and odonto-HPP.[1] Some of the recent European-wide genetic studies estimate the prevalence of the perinatal/infantile form at 1 in 300 000 persons, while the non-perinatal/infantile forms of HPP can be found in as high as 1 in 6370 persons.[2]

The clinical manifestations of HPP are heterogenous, leading from respiratory failure and neurological complications in severely affected children to mild dental issues in adults. Musculoskeletal pain and weakness, however, are the main manifestation in adults, often mimicking rheumatologic diseases.[3,4] Consequently, impaired mobility and reduced quality of life can be found in most patients.[5] Furthermore, HPP in adults is frequently associated with fractures such as subtrochanteric and metatarsal fractures as well as poor bone healing occurring in a setting of a fracture.[5]

Diagnosis of HPP is made by clinical and serological examinations and usually confirmed by genetic testing.[6] Due to the oftentimes variable disease presentation and mild symptoms, no formal guidelines for diagnosis of HPP existed. Recently, a set of diagnostic criteria was developed by the HPP International Working Group.[7] The leading biochemical hallmark of the condition is the low age- and sex-adjusted serum activity of the alkaline phosphatase (ALP), and consequently high levels of its substrates pyrophosphate (PPi), pyridoxal-5′-phosphate (PLP) or phosphoethanolamine (PEA), which can be measured in serum and urine, respectively. BMD measurements by DXA are not helpful for diagnosis and do not reflect the increased fracture risk in HPP.[8] Moreover, established and experimental bone turnover markers such as serum PINP, CTX, osteocalcin (OC), tartrate-resistant acid phosphatase 5b, or sclerostin do not differ between HPP and healthy individuals.[9]

Asfotase alfa, an enzyme replacement therapy (ERT), has been introduced for the treatment of childhood-onset HPP in recent years. It improves bone mineralization, respiratory function, and thus survival in severely affected children.[10] In adults, asfotase alfa therapy leads to normalization of bone mineralization, fracture healing, and improvement in pain and mobility.[11] Serum ALP excessively increases under asfotase alfa treatment and hence it is not useable for drug monitoring. In contrast, PEA, which is one of reliable parameters for diagnosis of HPP, can also be used for follow-up investigations under asfotase alfa treatment.[12,13] Parameters such as serum pyridoxal 5′-phosphate (PLP) to pyridoxal (PL) ratio also appear to be a better indicator for therapy adherence and effect of ERT than either PLP or urine PEA concentration alone.[14] Furthermore, serum and urine levels of PPi also appear to be a useful indicator for efficacy of ERT since their decrease under therapy is accompanied by beneficial effects on pseudofracture union.[15] Particularly, the urine pyrophosphate levels corrected by urine creatinine (uPPi/Cre) seem to be a good substitute for plasma PPi due to not requiring immediate filtration following the serum sampling.[15]

MicroRNAs (miRNAs) are a group of small non-coding ribonucleic acid (RNA) molecules, which are involved in posttranscriptional gene expression and they make up for 1% to 5% of the entire human genome.[16] miRNAs modulate gene expression through direct regulation of protein translation as well as influencing the stability of messenger RNA (mRNA) through cleavage or translational repression.[17] They regulate a vast variety of cell processes including cell differentiation, proliferation, expression of genes coding for growth factors and tumor suppressor proteins, metabolism as well as apoptosis.[17]

Recent studies have shown that circulating miRNAs are involved in the entirety of osteogenesis steps and they emphasize the genetic component in the multifactorial genesis of many bone diseases.[18,19] The role of these molecules in the development of bone diseases implies their potential use both as a novel therapy mechanism as well as a promising biomarker for monitoring the course of disease and therapy effectiveness.[19,20]

Previous studies suggested the ability of miRNAs in monitoring of bone-specific drugs. A differential expression of miRNAs was found under zoledronic acid and teriparatide treatment, respectively.[19,21] Other authors also reported about changes in miRNA profiles under teriparatide, zoledronate, alendronate, or denosumab over the time course of treatment.[22-24] Thus, specific miRNAs could serve as novel biological marker in the diagnostics and therapy of osteoporosis and potentially in other bone diseases. To our knowledge, this the first project dealing with miRNA changes in a patient with HPP and asfotase alfa treatment.

Case report

A 49-yr-old Caucasian woman presented at our osteo-endocrinological outpatient clinic at the age of 45 yr with childhood-onset HPP with mild to moderate symptoms during the childhood, which was genetically confirmed 1 yr prior to the first contact. The clinical presentation at an adult age involved primary musculoskeletal involvement with various manifestations. The patient suffered from atraumatic fractures, tooth loss, muscle cramps, recurrent myalgia, and bone pain as adult. The findings of genetic analysis revealed 2 heterozygote pathogenic variants in the TNSALP gene in exons 5: c346G > A [p(Ala116Thr)] and 12: c1363G > A [p.(Gly455Ser)]. The patient reported having HPP-related symptoms from early childhood, which included spontaneous loss of deciduous teeth and different dental health problems, among others, tooth loss in adulthood, with patient reporting having lost 8 teeth overall. At the timepoint of presentation, the patient indicated having chronic muscular weakness, recurrent myalgia with maximum intensity in the groins, as well as recurrent bone pain with the left upper extremity being most affected by it. Furthermore, the patient had a history of low-impact fractures, including a sub-trochanteric diaphyseal fracture of the left femur at the age of 41 after a fall from standing height in the metro. The fracture was treated by means of a dynamic hip screw and was followed by a 4-mo long rehabilitation period. The second fracture was diagnosed after the patient reported pain in the right thigh and it was classified as an insufficiency fracture. It involved a fissure in the ventrolateral aspect of femur cortex at the junction between the proximal and middle third of femur without signs of complete fracture of the femur shaft—typical for a pseudofracture associated with HPP. The fracture was not

treated surgically, and the patient instead received a course of extracorporeal shock wave therapy at one of the state-funded trauma centers without significant improvement. The last fracture, the left radius, took place at the age of 46. It was treated by means of plaster cask immobilization and subsequent physiotherapy.

Initial physical examination revealed a valgus deformity of both lower extremities, whereas the upper extremities revealed no signs of deformity. The patient reported recurrent falls from standing height with a frequency of approximately 5 times a year, due to unsteady and insecure gait without the use of walking aid, reflecting the substantial fracture risk. Further investigation revealed a presence of unspecific arthralgia without radiologic sings of chondrocalcinosis. Due to the prior misdiagnosis of osteoporosis at the age of 41, the patient received a single course of therapy with bisphosphonate drug ibandronate, which led to massive bone pain as reported by the patient. Due to chronic myalgia and bone pain, the patient reported using dexibuprofen and magnesium supplements sporadically. The use of corticosteroids, nicotine, and alcohol was negated by the patient. She reported having a regular menstrual cycle. In terms of patient's family history, her daughter was reported to have had numerous cardiac surgeries for aortic isthmus stenosis and structural heart valve defects. Furthermore, the patient's grandmother was said to have had similar symptoms as our patient.

BMD measurements by DXA 2.5 yr prior to the first presentation revealed normal BMD and TBS values. Ultrasound of the kidneys revealed no signs of nephrocalcinosis. The radiographic examination of the spine did not show vertebral fractures, but degenerative changes. The X-ray of the pelvis revealed incipient bilateral coxarthrosis. Finally, the radiographs of hips showed an intramedullary nail placed in the left femur at the transition from proximal to the middle third of the femur shaft with a presence of ventrally localized 6 mm thick cortical gradation with sings of osseous knitting after a prior femur fracture. On the other hand, the X-ray of the right femur showed an osseous fissure that presented itself as a 2 cm long ventrolateral linear translucency at the transition from proximal to middle third of femur shaft with an accompanying sclerosis, however, without a sign of complete osseous knitting (see Figure 1). Ophthalmological check was without pathological findings. Laboratory records showed low levels of ALP, low levels of fibroblast growth factor-23 (FGF-23), as well as a moderate vitamin D deficiency and elevated PLP levels (Table 1).

Finally, the patient was diagnosed with a genetically confirmed HPP with multiple musculoskeletal manifestations. Due to recurrent falls (approx. 5 times a yr) with low-traumatic fractures, very high risk of imminent fractures, incipient immobility, as well as increasing pain levels, an ERT with asfotase alfa at a dose of 1 milligram per kilogram of body weight 6 times a week was initiated. A specific treatment protocol for the treatment monitoring was constructed, based on the monitoring guidance for patients with HPP by Kishnani et al.[25] The clinical and laboratory examinations took place at baseline. In accordance with the internal protocol, analysis of miRNA expression levels, functionality tests, as well as physician- and patient-reported functionality scores were performed and investigated at the start of therapy and at different timepoints over the course of therapy.

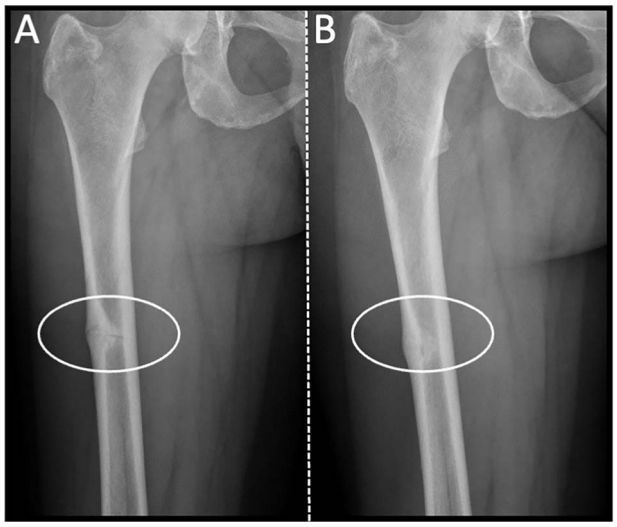

Figure 1. X-ray of the right femur performed at baseline showing the insufficiency fracture (A) and fracture union after 15 mo of treatment with asfotase alfa (B).

Materials and methods

Analysis of miRNA expression levels

An informed consent for experimentation with human subjects was obtained prior to collection of data and biological specimens. The patient's data were registered in the internal HPP biobank, and the patient was enrolled in a study on HPP, which was approved by the ethics committee of the City of Vienna on 04.09.2020 (EK 20-174-0820). All procedures were performed in compliance with relevant national and European Union laws on health data privacy.

miRNAs for the current study were selected based on miRNA discovery by small RNA-sequencing in 24 adult HPP patients and 24 healthy controls.[26] Informed consents were obtained from all participants. The study was approved by the ethical committee of the city of Vienna. The analysis of miRNA expression levels from serum samples was performed in a step-dependent manner using next generation sequencing (NGS) kits. miRNA extraction and analysis of circulating profiles in serum were analyzed at baseline, months 1, 2, 4, and 16.

We used an established workflow[27] including the following RNA/miRNA sampling kits for identification of the most significantly regulated ones: (1) miRNeasy® Mini Kit for RNA extraction (Qiagen®, Cat No. 217004), (2) RealSeq®-Biofluids NGS Library Preparation kit for miRNA and small RNA sequence analysis (RealSeq® Biosciences Inc., Cat No. 600-00048-SOM), (3) miND® spike-in quality control kit (TAmiRNA GmbH, Cat No. KT-5-041-MIND). Small RNA-sequencing libraries were analyzed on an Illumina NovaSeq 6000 SP1 flow cell (Cat No. 20028401), and raw data were processed using the miND® bioinformatic pipeline.[28]

We identified >800 miRNAs in patient serum by NGS analysis and selected a subset for further validation using the following criteria:

1. Tissue specificity index of greater than 0.7 as stated by Tissue Atlas of the Chair for Medical Bioinformatics of the University of Saarland[29]
2. Logarithmic fold change ($\log_2$FC) $>+0.8$ or <-0.8 or
3. Level of significance BH-adjusted $p<.05$

Table 1. Patient's blood sample findings performed at first contact in our osteo-endocrinological outpatient clinic.

Parameter	Laboratory value	Unit	Reference range
Hemoglobin	14.0	g/dL	13.0–16.0
Platelets	182	10^9/L	150–400
White blood cell count	8.2	10^9/L	4.00–10.00
C-reactive protein	**5.5**	**mg/L**	**<5.00**
Potassium	4.2	mmol/L	3.50–5.10
Sodium	141	mmol/L	136–146
Calcium (total)	2.61	mmol/L	2.20–2.65
Phosphate	1.29	mmol/L	0.81–1.45
Magnesium	0.82	mmol/L	0.77–1.03
Creatinine	0.56	mg/dL	0.51–0.95
Uric acid	3.6	mg/dL	2.60–6.00
Alkaline phosphatase	**20**	**U/L**	**30–120**
Glucose	88	mg/dL	70–100
Total protein	7.6	g/dL	6.6–8.3
Albumin	47.96	g/L	40.20–47.60
Beta-Crosslaps	0.20	ng/mL	0.03–0.44
Thyroid-stimulating hormone	0.75	μU/mL	0.38–5.33
Free T4	0.77	ng/dL	0.61–1.12
Parathyroid hormone (intact)	29	pg/mL	12–88
FGF-23	**15.1**	**pg/mL**	**23.2–95.4**
Osteocalcin	13.5	ng/mL	6.5–42.3
Vitamin D, 25-Hydroxy-	**44**	**nmol/L**	**75–250**
Vitamin B6	**>400**	**μg/L**	**5–30**

Out of >800 screened miRNAs, 84 miRNAs were significantly differently expressed between HPP and CTRLs in NGS analysis ($p<.05$). The most common expression sites of the 84 miRNAs as well as their occurrence in musculoskeletal tissue are shown in Table 2. Finally, the expression levels of the 84 validated miRNAs in reads per million (rpm) were logarithmized using the base of 10. Ultimately, the logarithmized expression levels were plotted against time axis, and we selected 6 miRNAs whose levels showed a clear linear trend of up- or downregulation with their logarithmized levels differing by at ≥50% compared to the baseline level.

Functionality tests

We used the following tests for the assessment of cardiovascular performance capacity and muscular strength:

1. Six-minute walking test (6-MWT)—a straightforward clinical test primarily used to asses functional exercise capacity and useful in evaluation of concomitant manifestations of extrapulmonary causes of chronic respiratory conditions such as sarcopenia, cardiovascular disease, cancer and frailty.[30] The 6-MWT was performed at baseline and month 10.
2. Chair raise test (CRT) (also known as five time chair raise test)—a well-established test for the assessment of leg power, primarily used to detect early changes in the age-related functionality status.[31] The CRT was performed at baseline and months 1 and 10.
3. Hand-grip dynamometry (HD)—a simple test utilizing hand-grip dynamometer assessing the strength of hand grip, whose decrease is linked to decrease in physical function, frailty as well as higher risk for loss of mobility, lean mass, and overall muscular strength.[32] The HD was performed at baseline and months 1, 2, 4, and 27.

Physician- and patient-reported questionnaires

Following physician- and patient-reported questionnaires regarding pain severity, improvement and disability were used:

1. Brief Pain Inventory Severity (BPI-S) and Brief Pain Inventory Interference (BPI-I)—self-administered scores, both being a part of one of the most used measures of the sensory and reactive aspects of pain. BPI has been used to assess pain across a range of conditions including musculoskeletal conditions, cancer, affective disorders, and post-surgery pain. Depending on the metrics investigated, it has been shown to have an acceptable to exquisite test–retest reliability, internal consistency, criterion, and construct validity as well as being reflective of and sensitive to change.[33]
2. Fatigue Severity Scale (FSS)—a self-reported scale, initially developed to asses fatigue in patients suffering from systemic lupus erythematosus and multiple sclerosis, currently having a wide range of applications in conditions such as RA, osteoarthritis (OA), and ankylosing spondylitis (ASI) as well as various long-term conditions including neurological diseases and cancer. FSS exhibits a good internal consistency, reliability, sensitivity to change, as well as construct and criterion validity.[34]
3. Patient Global Impression of Improvement (PGI-I)—the most frequently used scale for measuring patient's subjective response to treatment including a categorical scale rating patient's overall impression of their wellbeing since treatment initiation. It is used for variety of medical and psychiatric conditions, and it shows significant association with other instruments assessing depression and anxiety domains such as Symptom Checklist-90-Revised questionnaire and those assessing physical symptoms of pain such as Brief Pain Inventory Short Form.[35]

Table 2. List of 84 significantly regulated miRNAs, their most common expression site, reported expression in bone and/or skeletal muscle tissue, status as bone-/muscle specific vs non-specific and percent change of ≥50% after 16 mo compared to baseline (incl. a clear graphic trend).

microRNA	Most common expression site according to PHANTOM5 project[35]	Reported expression in bone and/or muscle tissue (+ reported / − not reported)	Bone and/or skeletal muscle specific (S-specific, NS-non-specific)	Percent change of ≥ 50% compared to baseline (incl. a clear graphic trend)
hsa-let-7i-3p	CD19+ B cell	+	NS	No
hsa-let-7i-5p	**Melanocyte**	**+ (Second most common site of expression**	**NS**	**Yes**
hsa-miR-1-3p	**Skeletal muscle cell**	**+**	**S (≤1 copy per million in other types of tissues)**	**Yes**
hsa-miR-101-3p	CD8+ T lymphocyte	+	NS	No
hsa-miR-103a-3p	Monocyte	+	NS	No
hsa-miR-107	Neural stem cell	+	NS	No
hsa-miR-10a-5p	Renal epithelial cell	+	NS	No
hsa-miR-122-3p	Hepatocyte	− in healthy bone or skeletal muscle tissue, low expression in osteosarcoma (1 copy per million)	N/A	No
hsa-miR-126-5p	Endothelial cell	+	NS	No
hsa-miR-128-3p	Neural stem cell	+	NS	No
hsa-miR-1285-3p	MCF7 breast cancer cell	+	NS	No
hsa-miR-1294	**Small airway epithelial cell**	**+**	**NS**	**Yes**
hsa-miR-1307-3p	Endothelial cell	+	NS	No
hsa-miR-130a-3p	Chorionic membrane cells	+	NS	No
hsa-miR-130b-3p	Embryonic stem cells	+	NS	No
hsa-miR-132-3p	Pituitary gonadotroph cell	+	NS	No
hsa-miR-139-3p	Hepatic sinusoidal endothelial cell	+, low (≤1 copy per million)	NS	No
hsa-miR-140-5p	Synoviocyte	+	NS	No
hsa-miR-141-3p	MCF7 breast cancer cell	+	NS	No
hsa-miR-142-3p	Natural killer cell	+	NS	No
hsa-miR-143-3p	Fibroblast	+	NS	No
hsa-miR-143-5p	Preadipocyte	+	NS	No
hsa-miR-144-3p	Amniotic membrane cell	+, low (≤1 copy per million)	NS	No
hsa-miR-144-5p	Neutrophil cell	+, low (≤2 copies per million)	NS	No
hsa-miR-145-3p	Synoviocyte	+	NS	No
hsa-miR-145-5p	Fibroblast	+	NS	No
hsa-miR-146a-5p	Melanocyte	+	NS	No
hsa-miR-146b-5p	Macrophage	+	NS	No
hsa-miR-148b-3p	CD14+ monocyte	+	NS	No
hsa-miR-155-5p	CD3+ T lymphocyte	+	NS	No
hsa-miR-15a-5p	CD19+ B lymphocyte	+	NS	No
hsa-miR-15b-3p	Endothelial cell	+	NS	No
hsa-miR-15b-5b	Neutrophil cell	+	NS	No
hsa-miR-16-2-3p	CD8+ T lymphocyte	+	NS	No
hsa-miR-16-5p	CD19+ B lymphocyte	+	NS	No
hsa-miR-181c-5p	Neural stem cell	+	NS	No
hsa-miR-185-5p	Melanocyte	+	NS	No
hsa-miR-18b-5p	Smooth muscle cell	− in healthy bone or skeletal muscle tissue, low expression in osteosarcoma (≤2 copies per million)	N/A	No
hsa-miR-190a-5p	Mesenchymal stem cell	+	NS	No
hsa-miR-191-5p	CD14+ monocyte	+	NS	No
hsa-miR-19a-3p	Urothelial cell	+	NS	No
hsa-miR-19b-3p	Urothelial cell	+	NS	No
hsa-miR-206	**Skeletal muscle cell**	**+**	**S (≤1 copy per million in other types of tissues)**	**Yes**
hsa-miR-22-3p	Endothelial cell	+	NS	No
hsa-miR-221-3p	Smooth muscle cell	+	NS	No
hsa-miR-221-5p	Smooth muscle cell	+	NS	No
hsa-miR-222-3p	Mesenchymal stem cell	+	NS	No
hsa-miR-23a-3p	Amniotic membrane cells	+	NS	No
hsa-miR-26a-5p	Neutrophil cell	+	NS	No
hsa-miR-26b-5p	Mast cell	+	NS	No
hsa-miR-28-3p	Induced pluripotent stem cell derived from dermal fibroblast	+	NS	No
hsa-miR-29a-3p	Smooth muscle cell	+	NS	No
hsa-miR-30a-3p	Renal cortical epithelial cell	+	NS	No

(*Continued*)

Table 2. Continued

microRNA	Most common expression site according to PHANTOM5 project[35]	Reported expression in bone and/or muscle tissue (+ reported / − not reported)	Bone and/or skeletal muscle specific (S-specific, NS-non-specific)	Percent change of ≥ 50% compared to baseline (incl. a clear graphic trend)
hsa-miR-30e-3p	CD14+ monocyte	+	NS	No
hsa-miR-30e-5p	Peripheral blood mononuclear cells (CD14+ monocytes and CD19+ B lymphocyte)	+	NS	No
hsa-miR-32-5p	CD14+ T lymphocyte	+	NS	No
hsa-miR-324-5p	Pituitary gland, MCF7 breast cancer cell	+	NS	No
hsa-miR-330-3p	Spinal cord	+	NS	No
hsa-miR-339-3p	CD8+ T lymphocyte	+	NS	No
hsa-miR-363-3p	Smooth muscle cell	+, low (≤8 copies per million in skeletal muscle cell and osteoblasts, higher in Saos-2 osteosarcoma cell line)	NS	No
hsa-miR-375-3p	**Corticothrops of the pituitary gland**	**+**	**NS**	**Yes**
hsa-miR-423-5p	Mesenchymal stem cell	+	NS	No
hsa-miR-4508	Smooth muscle cell	-	N/A	No
hsa-miR-451a	Neutrophil cell	+, low (≤6 copies per million in skeletal muscle cell and osteoblasts)	NS	No
hsa-miR-454-3p	Induced pluripotent stem cell derived from dermal fibroblast	+	NS	No
hsa-miR-486-5p	CD19+ B lymphocyte	+	NS	No
hsa-miR-500a-3p	Dendritic cell (monocyte)	+	NS	No
hsa-miR-502-3p	Alveolar epithelial cell	+	NS	No
hsa-miR-532-5p	Amniotic epithelial cell	+	NS	No
hsa-miR-545-5p	Smooth muscle cell	+, low (≤6 copies per million in skeletal muscle cell and osteoblasts)	NS	No
hsa-miR-548ad-5p	CD19+ B lymphocyte	-	N/A	No
hsa-miR-548ae-5p	Urothelial cell	+, low (≤4 copies per million in skeletal muscle cell and osteoblasts)	NS	No
hsa-miR-548ay-5p	Macrophage cell	− in healthy bone or skeletal muscle tissue, low expression in osteosarcoma (≤1 copy per million)	N/A	No
hsa-miR-548d-5p	Fibroblast	+, low (≤1 copy per million in and osteoblasts, ≤3 copies per million in Saos-2 osteosarcoma cell line)	NS	No
hsa-miR-576-3p	Bronchial epithelial cell	+	NS	No
hsa-miR-590-3p	Dendritic cell (monocyte)	+	NS	No
hsa-miR-624-5p	**Mesenchymal stem cell**	**+, low (≤7 copies per million in skeletal muscle cell and osteoblasts)**	**NS**	**Yes**
hsa-miR-625-5p	Smooth muscle cells	+	NS	No
hsa-miR-629-5p	H9 embryonic stem cell	+	NS	No
hsa-miR-652-3p	Retinal pigment epithelial cell	+	NS	No
hsa-miR-92a-3p	H9 embryonic stem cell	+	NS	No
hsa-miR-93-3p	H9 embryonic stem cell	+	NS	No
hsa-miR-95-3p	MCF7 breast cancer cell line	+, low (≤2 copies per million in skeletal muscle cells, not expressed in osteoblasts, ≤9 copies per million in Saos-2 osteosarcoma cell line)	NS	No
hsa-miR-96-5p	Pineal gland	+, low (≤15 copies per million in skeletal muscle cells, ≤1 copy per million in osteoblasts)	NS	No

4. Fibromyalgia Impact Questionnaire (FIQ)—a self-administered clinical instrument composed of 10 questions assessing the performance of tasks using large muscle groups, number of days feeling well and conversely the number of days feeling unfit for (physical) work and items assessing pain, fatigue, morning tiredness, anxiety, depression, stiffness, and work difficulty. It is used as an index of therapeutic efficacy in fibromyalgia-related research. It was shown to exhibit good sensitivity regarding therapeutic change, a credible construct validity as well as reliable test–retest features. Since its first publishing in 1991, it has undergone 2 modifications, which reflect the ever growing experience with the use of this instrument as well as incorporating a clarification for the scoring system.[36]
5. Western Ontario and McMaster University Hip Disability and Osteoarthritis Outcome (WOMAC) score—a reliable self-assessment, multidimensional, and widely used instrument for assessment of the severity of hip and knee OA that includes sub scores assessing 17 functional activities, 2 stiffness categories, and 5 pain-related activities. It displays a good internal consistency and satisfactory test–retest reliability, and it was shown to be superior to numerous other general and disease-specific measures of health status with being adapted trans-culturally and translated into over 60 languages, hence being used worldwide. It was also shown to reliably reflect numerous conditions other than knee or hip dysfunction and being indicative of psychological and constitutional status. Furthermore, it has been shown to be influenced by the presence of symptoms such as depression, lower back pain, fatigue, and symptom counts.[37]

Laboratory tests and radiographic imaging

The biochemical analyses (excluding miRNAs) included a baseline osteological assessment including complete blood count, C-reactive protein, potassium, sodium, calcium (total), phosphate, magnesium, creatinine, uric acid, ALP, glucose, total protein, albumin, C-terminal telopeptide of type 1 collagen also known as beta-crosslaps (CTX), thyroid-stimulating hormone, free tetraiodothyronine (T4), parathyroid hormone (intact), FGF-23, OC, 25OHD, and vitamin B6 as measured by levels of PLP.

The DXA measurements were performed using the GE Healthcare Lunar Prodigy device (GE Healthcare). Standard radiographs were performed using a Philips DigitalDiagnost radiography system (Philips).

Results

Longitudinal course of circulating miRNAs

Over the time course of 400 days, several miRNAs significantly changed over the course of the treatment with asfotase alfa. Visual inspection identified a clear trend of down-/upregulation together with a relative change in expression (rpm) of at least 50% in 6 miRNAs. The identification of the most common expression sites and classification as bone-/muscle-specific was performed using the data from the functional annotation of the mammalian genome project FANTOM-5 at the RIKEN institute of physical and chemical research in Japan.[38] *Hsa-let-7i-5p* was shown to be expressed in numerous tissue types, hence being non-specific with regards to bone and muscle tissue. *Hsa-let-7i-5p* in patient's serum increased from 20 000 rpm to 30 000 (50.00%). *Hsa-miR-1-3p* displayed a reduction from 60 rpm down to 20 rpm after 400 days of treatment (66.66%) and it was shown to be primarily expressed in skeletal muscle cells and mesodermal cells hence leading to its classification as bone-/muscle-specific miRNA. *Hsa-miR-1294* exhibited an increase in expression from 55 rpm to 90 (63.63%). It was most commonly expressed by small airway epithelial cells, and it was classified as non-specific for bone/muscle tissue. The fourth identified miRNA—*hsa-miR-206* was identified as muscle-/bone-specific with skeletal muscle cells and myoblasts being its primary expression site and its expression being downregulated from 187 rpm to 27 rpm (85.57%). *Hsa-miR-375-3p* exhibited a downregulation from 70 rpm to 20 rpm (71.43%) and was shown to be bone-/muscle non-specific and mostly expressed in pituitary and pineal glands. However, it was shown by other researchers to be involved in the regulation of osteogenesis in different conditions. Finally, *hsa-miR-624-5p* showed an upregulation from 36 rpm to 61 rpm (69.44%) likewise being classified as bone/muscle non-specific and being expressed in various cell types. The change in expression levels of the 6 miRNAs identified is shown in Figure 2.

Functionality tests and physician- and patient-reported questionnaires

All the scores analyzed showed an improvement at 16 mo when compared to baseline. Two functionality scores, CRT and 6-MWT, were, however, analyzed at 10 mo post treatment initiation. The percentual change in the values of the scores at the end of observation period compared to the baseline is shown in brackets. The BPI-S showed a reduction from 7.3 to 3 (58.62%). BPI-I exhibited a reduction from 5.9 to 1.9 (68.29%). The FSS score at 16 mo displayed a value of 3.1 compared to 4.7 at baseline (33.33%). The PGI-I score displayed a value of 1 at 16 mo compared to the baseline score of 4 (75.00%). Likewise, the WOMAC score exhibited a reduction from 42 points to 15.4 at the end of surveillance period (63.29%). The FIQ displayed a reduction with its value showing a decrease from 29.3 to 16.7 (43.02%). The CRT displayed an improvement with the patient performing it in 8.5 s, down from 15.3 s at the baseline (44.46%). The patient succeeded in walking 479 m in 6 min at 10 mo compared to 358 m at baseline (33.89%). In terms of muscular strength, as measured by hand grip dynamometry, the patient's hand grip strength of the right/left hand increased from 24 kg/17 kg at baseline to 27 kg/21 kg at 27 mo (+12.50%/+23.53%). The results of functionality tests and symptom-related scores are shown in Figures 3 and 4.

Discussion

This case report highlights a potential link between the expression levels of 2 circulating muscle-specific miRNAs—*hsa-miR-1-3p* and *hsa-miR-206* and functional/symptom improvements in an adult patient treated with asfotase alfa for childhood-onset HPP and multiple atraumatic fractures. Both miRNAs showed a clear pattern of downregulation over the course of treatment with asfotase alfa with at least 50% reduction of the baseline levels of circulating miRNA. Their downregulation also coincided with a clear

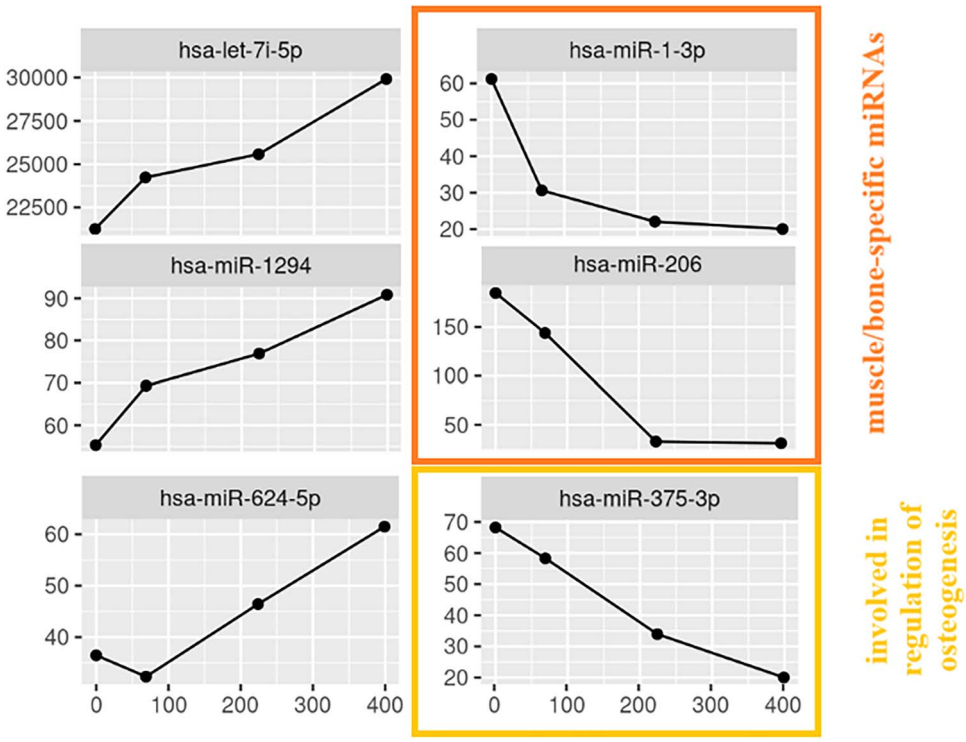

Figure 2. Change in expression levels of the 6, most significantly regulated miRNAs (in reads per million) over the course of therapy (in days) (muscle/bone-specific miRNAs and ones involved in regulation of osteogenesis highlighted).

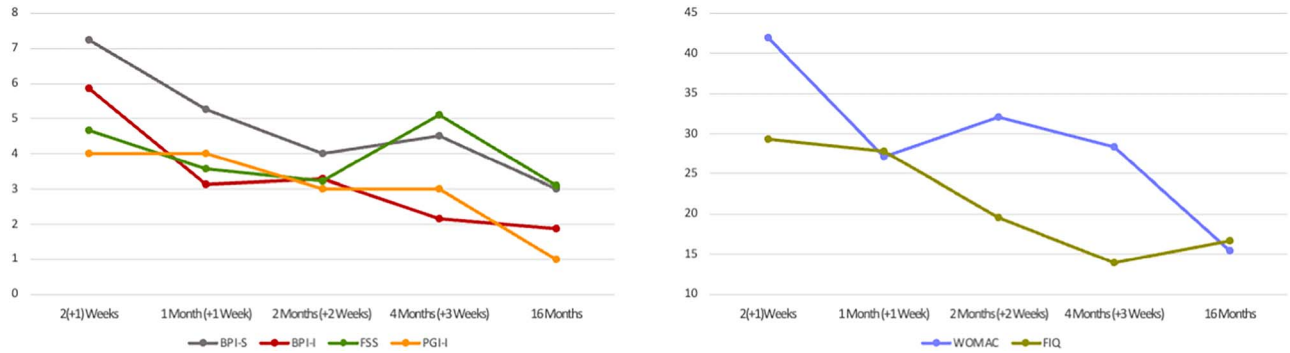

Figure 3. BPI-S, BPI-I, FSS, and PGI-I scores over the course of treatment (left); WOMAC and FIQ scores over the course of treatment (right).

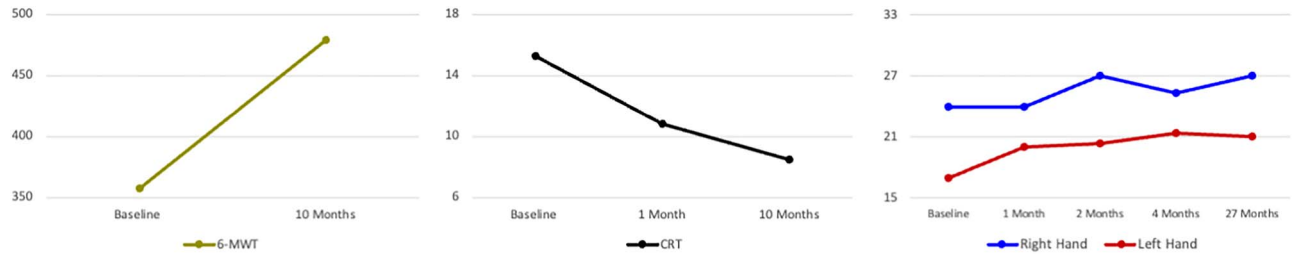

Figure 4. 6-MWT (in meters) (left); CRT (in seconds) (middle); and hand grip strength measured by hand grip dynamometry (in kilograms, right) over the course of treatment.

reduction in time needed to perform the CRT and increase in the distance walked during the 6-MWT. Moreover, the improvement in patient's functionality in activities of daily life was also accompanied by significant reductions in the symptom-oriented scores such as BPI-S, BPI-I, FSS, and PGI-I scores. Furthermore, the 2 questionnaires designed to assess the function of joints and muscles—WOMAC and FIQ also showed a clear reduction pattern in the score after period of 16 mo under therapy with asfotase. This could point to potential role of *hsa-miR-1-3p* and *hsa-miR-206* in complex signaling mechanisms in bone and muscle tissue, which are likely altered in patients with HPP.

A study by Yin et al. has identified *hsa-miR-1-3p* and *hsa-miR-206* as one of the 4 miRNAs altered in acute prolonged exercise, correlated with an increase in the established biomarkers such as myoglobin, cardiac troponin I, and

creatine kinase MB isoenzyme. *Hsa-miR-1-3p* was shown to be acutely elevated in 18 individuals after a prolonged endurance run; however, its level returned to normal 24 h after exercise. *Hsa-miR-206* levels were shown to be decreased at 24 h post exercise. Furthermore, the change in *hsa-mir-1-3p* levels was correlated with myoglobin levels at the 24-h mark, the latter also showing an acute elevation after a prolonged exercise. This points to the possible role of the 2 miRNAs in muscle tissue signaling and their physiological elevation in plasma after acute prolonged exercise. Similarly, a study by Chalcat et al. demonstrated that the increase in *hsa-miR-1-3p*, creatine kinase, and myoglobin levels after exercise was less profound after a second exercise session 2 wk after the initial one. The maximal voluntary contraction torque deficit, which was an indicator of adaptiveness of neuromuscular system to cell damage caused by strenuous exercise, was shown to correlate among others with the aforementioned biomarkers.[39]

hsa-miR-1-3p was also suggested as a key regulator in bone metabolism in postmenopausal osteoporosis through its targeting of mRNA coding for RAS oncogene family RAB5C[40] and their transition from osteogenic to adipogenic differentiation of human mesenchymal stem cells (hMSCs) in the development of osteoporosis.[41]

Hu et al. have demonstrated that muscle-specific miRNAs *hsa-miR-1*, *hsa-miR-133*, and *hsa-miR-206* are 2 to 4-fold more expressed in serum of children with Duchenne muscular dystrophy, a neuromuscular disease whose hallmark is muscular weakness. The study also showed that the levels of the 3 miRNAs correlated inversely with muscular strength, muscular function, as well as quality of life.[42] Research by Koutsolidou et al. has shown the role of *hsa-miR-1*, *hsa-miR-133a*, *hsa-miR-133b*, and *hsa-miR-206* in the later stages of fetal development with the increase in their circulating levels being proportional to the capacity of myotube formation by myoblast. Furthermore, it was shown that the ectopic expression of the inducer of the 4 miRNAs—MyoD causes an induction in muscle cell differentiation as well pointing out to their role in regulation in muscle formation.[43] Toklowicz et al. have demonstrated that *hsa-miR-486–5p*, *hsa-miR-191–5p*, and *hsa-miR-133a-3p* are among the most stable muscle-specific miRNAs and should hence be used as reference for normalization of RT-qPCR data in muscle tissues.[44]

Hsa-miR-206 was also shown by Lu et al. to target histone deacetylase 4 gene and thereby regulate the proliferation and apoptosis of osteoblast cells in vitro. Furthermore, serum *hsa-miR-206* levels in postmenopausal women were significantly lower when compared to controls, also showing a positive correlation with BMD.[45] A study by Chen et al. investigated the role of *hsa-miR-206* in osteoblast differentiation and showed that its levels were significantly reduced in the bone marrow hMSCs during osteogenic induction during the days 7 and 14. Conversely, the hMSCs overexpressing *hsa-miR-206* showed reduced activity of ALP and reduced OC secretion.[46] The results of the study by Chen et al. correspond to the baseline laboratory findings from our patient's serum that showed the elevated expression of *hsa-miR-206* and low levels of ALP, the latter being pathognomonic of HPP. During the treatment with recombinant form of TNSALP, a significant downregulation of *hsa-miR-206* was identified, which corresponds to the increased enzyme activity, suggestive of interaction between this miRNA and ALP.

Although not primarily expressed in musculoskeletal tissues, *hsa-miR-375-3p* was shown to be involved in regulation of osteogenesis. Sun et al. have investigated the role of downstream signaling targets of *hsa-miR-375-3p* including low-density lipoprotein receptor-related protein 5 (LRP5), which is a co-receptor involved in β-catenin and Wnt signaling pathways. It was shown that mice with silenced LRP5 gene had elevated level of this miRNA, and the results of microCT-measurements revealed looser and inferiorly connected bone trabeculae.[47] Patients with osteoporosis displayed higher levels of *hsa-miR-375-3p*, which tend to gradually decrease through prolongation of osteogenic differentiation in hMSCs. The researchers demonstrated that the therapy with teriparatide elevated the osteogenic capacity and mineralization potential of the hMSCs and were shown to be inhibited through overexpression of *hsa-miR-375-3p*.[48]

Although PEA and PPi are reliable markers for the follow-up of asfotase alfa treatment, they are not available in Austria and were therefore not used. The role of PLP in the diagnosis of HPP is undisputed.[7] However, the interpretation of PLP under ERT is difficult and is therefore not regularly measured during follow-up examinations at our institute. However, apart from ALP, the established laboratory values did not change during therapy. If miRNAs are more sensitive than established markers such as PLP, PPi, or PEA are currently unclear.

In summary, our patient suffered from childhood-onset HPP and displayed atraumatic fractures, tooth loss, muscle spasms, myalgias, and bone pain as adult. Of the 6 miRNAs that showed significant changes in their expression levels, 2 miRNAs were identified as bone and muscle specific (hsa-miR-1-3p and hsa-miR-206) and one miRNA involved in the regulation of osteogenesis (hsa-miR-375-3p). This could be different in patients with other symptoms such as renal or neurological diseases or respiratory failure. Future studies need to investigate miRNAs in larger cohorts to show whether miRNAs can also reflect changes in other organ systems.

Conclusion

miRNA profiles change over the course of therapy with asfotase alfa, accompanying improvements in functionality and quality of life scores. Muscle-specific miRNAs *hsa-miR-1-3p* and *hsa-miR-206* could potentially be used as a novel biomarker for monitoring of treatment in HPP patients. Furthermore, *hsa-miR-375* could serve as an indicator of altered regulation of osteogenic processes in HPP patients. However, prospective studies with greater sample size are needed to elucidate potential role of the 3 miRNAs in pathogenesis of HPP and validate their prognostic role as a biomarker in HPP.

Author contributions

Benjamin Hadzimuratovic (Conceptualization, Methodology, Formal Analysis, Investigation, Resources, Writing—Original Draft, Visualization, Project Administration), Judith Haschka (Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Resources, Data Curation, Writing—Original Draft, Writing—Review & Editing, Visualization, Supervision, Project administration, Funding acquisition), Matthias Hackl (Software, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing—Review & Editing, Visualization), Andreas B. Diendorfer (Software, Validation, Formal analysis, Investigation, Resources, Data Curation, Writing—Review & Editing,

Visualization), Andreas Mittelbach (Investigation, Data Curation, Writing—Review & Editing), Julia Feurstein (Investigation, Data Curation, Writing—Review & Editing), Jochen Zwerina (Conceptualization, Resources, Writing—Review & Editing, Supervision, Project Administration, Funding Acquisition), Heinrich Resch (Conceptualization, Writing—Review & Editing, Supervision, Project Administration), and Roland Kocijan (Conceptualization, Methodology, Resources, Data Curation, Writing—Original Draft, Writing—Review & Editing, Visualization, Supervision, Project administration, Funding acquisition)

Funding

This case report was a part of a greater research project on HPP supported by Alexion Pharmaceuticals Inc., a subsidiary of AstraZeneca [ISR 100252]. Alexion Pharmaceuticals Inc. was not involved in study design, data collection or interpretation of data. Funds provided were used in the analysis of biological specimens.

Conflicts of interest

J.H. received speaker honoraria from Alexion Pharmaceuticals Inc. H.R. received speaker honoraria from Alexion Pharmaceuticals Inc. R.K. received financial support for attending medical congresses, speaker honoraria and participated in ad boards, sponsored by Alexion Pharmaceuticals Inc. This study was supported by Alexion with a research grant. M.H. and A.D. receive salary from TAmiRNA GmbH. MH holds stock options in TAmiRNA GmbH.

Data availability

The data underlying this article are available in *medRxiv*, at: http://medrxiv.org/content/early/2024/07/17/2024.07.17.24310437.abstract

References

1. Whyte MP. Hypophosphatasia-aetiology, nosology, pathogenesis, diagnosis and treatment. *Nat Rev Endocrinol*. 2016;12(4):233–246. https://doi.org/10.1038/nrendo.2016.14
2. Mornet E, Yvard A, Taillandier A, Fauvert D, Simon-Bouy B. A molecular-based estimation of the prevalence of hypophosphatasia in the European population. *Ann Hum Genet*. 2011;75(3):439–445. https://doi.org/10.1111/j.1469-1809.2011.00642.x
3. Feurstein J, Behanova M, Haschka J, et al. Identifying adult hypophosphatasia in the rheumatology unit. *Orphanet J Rare Dis*. 2022;17(1):435–438.
4. Dahir KM, Kishnani PS, Martos-Moreno GÁ, et al. Impact of muscular symptoms and/or pain on disease characteristics, disability, and quality of life in adult patients with hypophosphatasia: a cross-sectional analysis from the global HPP registry. *Front Endocrinol*. 2023;14(March 2023):1–9. https://doi.org/10.3389/fendo.2023.1138599
5. Khan AA, Josse R, Kannu P, et al. Hypophosphatasia: Canadian update on diagnosis and management. *Osteoporos Int*. 2019;30(9):1713–1722. https://doi.org/10.1007/s00198-019-04921-y
6. Riancho JA. Diagnostic approach to patients with low serum alkaline phosphatase. *Calcif Tissue Int*. 2023;112(3):289–296. https://doi.org/10.1007/s00223-022-01039-y
7. Khan AA, Brandi ML, Rush ET, et al. Hypophosphatasia diagnosis: current state of the art and proposed diagnostic criteria for children and adults. *Osteoporos Int*. 2024;35(3):431–438.
8. Hepp N, Frederiksen AL, Duno M, Jørgensen NR, Jensen JEB. Biochemical and clinical manifestations in adults with hypophosphatasia: a national cross-sectional study. *Osteoporos Int*. 2022;33(12):2595–2605. https://doi.org/10.1007/s00198-022-06536-2
9. Hepp N, Folkestad L, Møllebæk S, et al. Bone-microarchitecture and bone-strength in a sample of adults with hypophosphatasia and a matched reference population assessed by HR-pQCT and impact microindentation. *Bone*. 2022;160(116420):116420. https://doi.org/10.1016/j.bone.2022.116420
10. Whyte MP, Greenberg CR, Salman NJ, et al. Enzyme-replacement therapy in life-threatening hypophosphatasia. *N Engl J Med*. 2012;366(10):904–913. https://doi.org/10.1056/NEJMoa1106173
11. Kishnani PS, Rockman-Greenberg C, Rauch F, et al. Five-year efficacy and safety of asfotase alfa therapy for adults and adolescents with hypophosphatasia. *Bone*. 2019;121(121):149–162.
12. Shajani-Yi Z, Ayala-Lopez N, Black M, Dahir KMC. Urine phosphoethanolamine is a specific biomarker for hypophosphatasia in adults. *Bone*. 2022;163(July):116504.
13. Seefried L, Rak D, Petryk A, Genest F. Bone turnover and mineral metabolism in adult patients with hypophosphatasia treated with asfotase alfa. *Osteoporos Int*. 2021;32(12):2505–2513. https://doi.org/10.1007/s00198-021-06025-y
14. Akiyama T, Kubota T, Ozono K, et al. Pyridoxal 5′-phosphate and related metabolites in hypophosphatasia: effects of enzyme replacement therapy. *Mol Genet Metab*. 2018;125(1–2):174–180.
15. Hidaka N, Murata H, Tachikawa K, et al. The effect of asfotase alfa on plasma and urine pyrophosphate levels and pseudofractures in a patient with adult-onset hypophosphatasia. *JBMR Plus*. 2023;7(12):1–8. https://doi.org/10.1002/jbm4.10842
16. MacFarlane L-A, Murphy PR. MicroRNA: biogenesis, function and role in cancer. *Curr Genomics*. 2010;11(7):537–561. https://doi.org/10.2174/138920210793175895
17. Bartel DP. Metazoan microRNAs. *Cell*. 2018;173(1):20–51.
18. Roberts SB, Wootton E, De Ferrari L, Albagha OM, Salter DM. Epigenetics of osteoarticular diseases: recent developments. *Rheumatol Int*. 2015;35(8):1293–1305. https://doi.org/10.1007/s00296-015-3260-y
19. Grillari J, Mäkitie RE, Kocijan R, et al. Circulating miRNAs in bone health and disease. *Bone*. 2021;145(September 2020):115787. https://doi.org/10.1016/j.bone.2020.115787
20. Hackl M, Semmelrock E, Grillari J. The role of microRNAs in osteoporosis diagnostics. *Osteologie*. 2021;30(03):222–229. https://doi.org/10.1055/a-1514-1800
21. Kocijan R, Weigl M, Skalicky S, et al. MicroRNA levels in bone and blood change during bisphosphonate and teriparatide therapy in an animal model of postmenopausal osteoporosis. *Bone*. 2020 Feb;131:115104.
22. Anastasilakis AD, Makras P, Pikilidou M, et al. Changes of circulating MicroRNAs in response to treatment with teriparatide or denosumab in postmenopausal osteoporosis. *J Clin Endocrinol Metab*. 2018;103(3):1206–1213. https://doi.org/10.1210/jc.2017-02406
23. Li J, Li Y, Wang S, Che H, Wu J, Ren Y. miR-101-3p/Rap1b signal pathway plays a key role in osteoclast differentiation after treatment with bisphosphonates. *BMB Rep*. 2019;52(9):572–576. https://doi.org/10.5483/BMBRep.2019.52.9.076
24. Messner Z, Vázquez DC, Haschka J, et al. Circulating miRNAs respond to Denosumab treatment after 2 years in postmenopausal women with osteoporosis—the MiDeTe study. *J Clin Endocrinol Metab*. 2023;108(5):1154–1165.
25. Kishnani PS, Rush ET, Arundel P, et al. Monitoring guidance for patients with hypophosphatasia treated with asfotase alfa. *Mol Genet Metab*. 2017;122(1–2):4–17.
26. Haschka J, Messner Z, Feurstein J, et al. Circulating microRNAs in patients with hypophosphatasia results of the first micro-RNA analysis in HPP. medRxiv [Internet] 2024 Jan 1;2024.07.17.24310437. http://medrxiv.org/content/early/2024/07/17/2024.07.17.24310437.abstract
27. Khamina K, Diendorfer AB, Skalicky S, et al. A MicroRNA next-generation-sequencing discovery assay (miND) for genome-scale analysis and absolute quantitation of circulating microRNA biomarkers. *Int J Mol Sci*. 2022;23(3):1226. https://doi.org/10.3390/ijms23031226
28. Diendorfer A, Khamina K, Pultar M, Hackl M. miND (miRNA NGS discovery pipeline): a small RNA-seq analysis pipeline and report generator for microRNA biomarker discovery studies

[version 1; peer review: 2 approved with reservations]. *F1000Research*. 2022;11:233. https://doi.org/10.12688/f1000research.94159.1

29. Ludwig N, Leidinger P, Becker K, et al. Distribution of miRNA expression across human tissues. *Nucleic Acids Res*. 2016;44(8):3865–3877. https://doi.org/10.1093/nar/gkw116
30. Agarwala P, Salzman SH. Six-minute walk test: clinical role, technique, coding, and reimbursement. *Chest*. 2020;157(3):603–611.
31. Hellmers S, Fudickar S, Lau S, et al. Measurement of the chair rise performance of older people based on force plates and IMUs. *Sensors*. 2019;19(6):1–22. https://doi.org/10.3390/s19061370
32. Benton MJ, Spicher JM, Silva-Smith AL. Validity and reliability of handgrip dynamometry in older adults: a comparison of two widely used dynamometers. *PLoS One*. 2022;17(6):e0270132.
33. Stanhope J. Brief pain inventory review. *Occup Med*. 2016;66(6):496–497. https://doi.org/10.1093/occmed/kqw041
34. Hewlett S, Dures E, Almeida C. Measures of fatigue: Bristol rheumatoid arthritis fatigue multi-dimensional questionnaire (BRAF MDQ), Bristol rheumatoid arthritis fatigue numerical rating scales (BRAF NRS) for severity, effect, and coping, Chalder fatigue questionnaire (CFQ), checklist. *Arthritis Care Res*. 2011;63(SUPPL. 11):S263–86. https://doi.org/10.1002/acr.20579
35. Demyttenaere K, Desaiah D, Petit C, Croenlein J, Brecht S. Patient-assessed versus physician-assessed disease severity and outcome in patients with nonspecific pain associated with major depressive disorder. *Prim Care Companion J Clin Psychiatry*. 2009;11(1):08–15. https://doi.org/10.4088/PCC.08m00670
36. Bennett R. The fibromyalgia impact questionnaire (FIQ): a review of its development, current version, operating characteristics and uses. *Clin Exp Rheumatol*. 2005;23(5 SUPPL. 39):S154–S162.
37. Wolfe F. Determinants of WOMAC function, pain and stiffness scores: evidence for the role of low back pain, symptom counts, fatigue and depression in osteoarthritis, rheumatoid arthritis and fibromyalgia. *Rheumatology*. 1999;38(4):355–361. https://doi.org/10.1093/rheumatology/38.4.355
38. Lizio M, Harshbarger J, Shimoji H, et al. Gateways to the FANTOM5 promoter level mammalian expression atlas. *Genome Biol*. 2015;16(1):1–14. https://doi.org/10.1186/s13059-014-0560-6
39. Chalchat E, Martin V, Charlot K, et al. Circulating microRNA levels after exercise-induced muscle damage and the repeated bout effect. *Am J Physiol Regul Integr Comp Physiol*. 2023;324(1):R58–R69. https://doi.org/10.1152/ajpregu.00096.2022
40. Wang R, Lu A, Liu W, et al. Searching for valuable differentially expressed miRNAs in postmenopausal osteoporosis by RNA sequencing. *J Obstet Gynaecol Res*. 2020;46(7):1183–1192. https://doi.org/10.1111/jog.14307
41. Du G, Cheng X, Zhang Z, et al. TGF-Beta induced key genes of osteogenic and Adipogenic differentiation in human mesenchymal stem cells and MiRNA–mRNA regulatory networks. *Front Genet*. 2021;12(November):1–13. https://doi.org/10.3389/fgene.2021.759596
42. Hu J, Kong M, Ye Y, Hong S, Cheng L, Jiang L. Serum miR-206 and other muscle-specific microRNAs as non-invasive biomarkers for Duchenne muscular dystrophy. *J Neurochem*. 2014;129(5):877–883. https://doi.org/10.1111/jnc.12662
43. Koutsoulidou A, Mastroyiannopoulos NP, Furling D, Uney JB, Phylactou LA. Expression of miR-1, miR-133a, miR-133b and miR-206 increases during development of human skeletal muscle. *BMC Dev Biol*. 2011;11(1):1–9. https://doi.org/10.1186/1471-213X-11-34
44. Tokłowicz M, Żbikowska A, Janusz P, Kotwicki T, Andrusiewicz M, Kotwicka M. MicroRNA expression profile analysis in human skeletal muscle tissue: selection of critical reference. *Biomed Pharmacother*. 2023;162(April):114682. https://doi.org/10.1016/j.biopha.2023.114682
45. Lu Z, Wang D, Wang X, Zou J, Sun J, Bi Z. MiR-206 regulates the progression of osteoporosis via targeting HDAC4. *Eur J Med Res*. 2021;26(1):8–7.
46. Chen Y, Yang YR, Fan XL, et al. MiR-206 inhibits osteogenic differentiation of bone marrow mesenchymal stem cells by targetting glutaminase. *Biosci Rep*. 2019;39(3):1–9. https://doi.org/10.1042/BSR20181108
47. Sun T, Li CT, Xiong L, et al. miR-375-3p negatively regulates osteogenesis by targeting and decreasing the expression levels of LRP5 and β-catenin. *PLoS One*. 2017;12(2):1–16. https://doi.org/10.1371/journal.pone.0171281
48. Lei N, Liang X, Wang P, Liu Q, Wang W. Teriparatide alleviates osteoporosis by promoting osteogenic differentiation of hMSCs via miR-375 / RUNX2 axis. *Eur Rev Med Pharmacol Sci*. 2019;23(24):11043–11050. https://doi.org/10.26355/eurrev_201912_19812