

# Week-by-week changes in serum levels of bone-related circulating microRNAs and bone turnover markers

Patryk Zarecki<sup>1</sup>, Fatma Gossiel<sup>1</sup>, Johannes Grillari<sup>2,3,4</sup>, Miguel Debono<sup>1</sup>, Matthias Hackl<sup>4,5</sup>, Richard Eastell<sup>1,\*</sup>

<sup>1</sup>School of Medicine and Population Health, Division of Clinical Medicine, University of Sheffield, S10 2RX Sheffield, United Kingdom

<sup>2</sup>Department of Biotechnology, Institute of Molecular Biotechnology, University of Natural Resources and Life Sciences, 1180 Vienna, Austria

<sup>3</sup>Ludwig Boltzmann Institute for Traumatology, the Research Center in Cooperation with AUVA, 1200 Vienna, Austria

<sup>4</sup>Austrian Cluster for Tissue Regeneration, 1200 Vienna, Austria

<sup>5</sup>TAmiRNA GmbH, 1110 Vienna, Austria

\*Corresponding author: Richard Eastell, Division of Clinical Medicine, University of Sheffield, Medical School, Beech Hill Road, Sheffield S10 2RX, United Kingdom (r.eastell@sheffield.ac.uk).

## Abstract

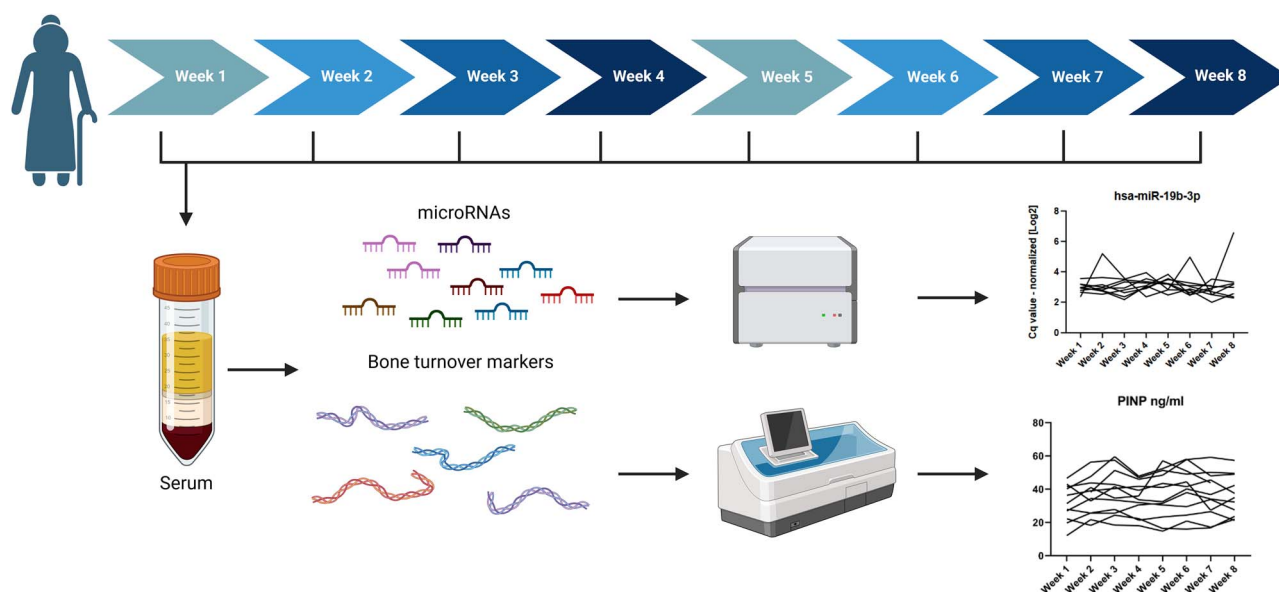
MicroRNAs are involved in post-transcriptional regulation of gene expression. Due to their regulatory role, microRNAs are differently expressed during specific conditions in healthy and diseased individuals, so microRNAs circulating in the blood could be used as diagnostic and prognostic biomarkers for various diseases and conditions. We want to investigate the variability of circulating microRNAs and bone turnover markers in weekly time intervals in older women. In a single-site longitudinal study, a panel of 19 bone-related miRNAs was measured using the osteomiR RT-qPCR assay in serum samples of 35 postmenopausal women divided into 3 groups: healthy controls ( $n = 12$ ), low BMD ( $n = 14$ ), and vertebral fractures ( $n = 9$ ). Blood samples for measurement of CTX, PINP, OC, and bone ALP were collected once per week for 8 weeks at 9:00 AM after overnight fasting. Serum samples from all participants were analyzed for 19 microRNA bone biomarkers and 4 bone turnover markers over 8 weeks. We analyzed the data using a mixed model analysis of variance and found no significant changes between week-by-week time points in any of the groups. To estimate intraindividual variability between weekly time points, we have calculated the median coefficient of variation (CV). This was between 28.4% and 80.2% for microRNA, with an assay CV of 21.3%. It was between 8.5% and 15.6% for bone turnover markers, with an assay CV of 3.5% to 6.5%. The intraindividual variability was similar between groups. Circulating microRNAs measured in serum had a higher weekly intraindividual variability than bone turnover markers due in part to a higher assay CV.

**Keywords:** circulating microRNA, bone turnover markers, variability, osteoporosis, biomarkers, RT-qPCR

## Lay Summary

MicroRNAs regulate cell activity, and they circulate in the bloodstream. If we can be sure that they do not show much week-to-week variability, we can monitor treatment response in diseases such as osteoporosis. We tested this by measuring microRNAs in 35 women who had blood drawn once a week for 8 weeks. We found that the 19 microRNAs we measured all showed significant variability, which might limit their use in clinical practice.

## Graphical Abstract



Received: December 4, 2023. Revised: February 27, 2024. Accepted: March 11, 2024

© The Author(s) 2024. Published by Oxford University Press on behalf of the American Society for Bone and Mineral Research.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<https://creativecommons.org/licenses/by/4.0/>), which permits unrestricted reuse, distribution, and reproduction in any medium, provided the original work is properly cited.

## Introduction

MicroRNAs (miRNAs) play a critical role in post-transcriptional regulation of gene expression. Due to the regulatory role, miRNAs are differently expressed during certain conditions in healthy and diseased individuals and could be used as diagnostic and prognostic biomarkers for various diseases and conditions. Previously published data have shown that some miRNAs can affect bone homeostasis, including bone remodeling and fracture healing, by altering the gene expression in osteoblasts, osteoclasts, and osteocytes.<sup>1</sup> More than 2000 human miRNAs have so far been reported,<sup>2</sup> which are transcribed in the nucleus as single-stranded RNAs, processed, and exported into the cytoplasm, where they are incorporated in the RNA-induced silencing complex to regulate gene expression. However, the active or passive release of miRNAs from cells within extracellular vesicles or bound to specific proteins results in a stable presence of miRNAs outside of cells, allowing the use of blood serum, urine, or saliva for detection.<sup>3</sup> Such “circulating” miRNAs have been suggested as a promising source of minimal-invasive biomarkers<sup>1</sup> in bone health and disease. Recent publications have considered the use of circulating miRNAs in fracture prediction and monitoring of osteoporosis treatment, as well as their use in secondary osteoporosis and rare bone disease.<sup>1</sup>

When making serial measurements of an analyte, it must show low day-to-day or week-to-week within-subject variability with the expected change. This issue of signal-to-noise has been studied extensively for bone turnover markers. For example, in monitoring alendronate therapy for osteoporosis, this ratio is highest for bone resorption markers, bone formation markers, and bone mineral density (BMD).<sup>4</sup> In the EuBIVAS study, 91 subjects were studied weekly for 10 weeks, and the within-subject variability for CTX and PINP was 15% and 9%, respectively.<sup>5</sup> The favorable ratio of signal to noise for bone turnover markers allows the identification of responders to bisphosphonate therapy in osteoporosis, as shown in the TRIO study,<sup>6</sup> and this led the International Osteoporosis Foundation and International Federation of Clinical Chemistry and Laboratory Medicine (International Osteoporosis Foundation (IOF)/International Federation of Clinical Chemistry and Laboratory Medicine (IFCC)) to recommend the use of CTX and PINP for identifying poor adherence in patients started on oral bisphosphonate therapy for osteoporosis.<sup>7</sup>

We do not know whether the miRNA that are under study for osteoporosis have low variability and therefore likely to be useful in monitoring changes due to therapy. We need to know this for patients with osteoporosis (eg, low BMD or the presence of vertebral fracture) for our treatment studies and for women with normal BMD (for our prevention studies). There are limited data on the variability of miRNA in serum, and these tended to show large variability.<sup>8,9</sup> There are no studies on osteoporosis. In the current study, we investigated week-to-week variability of miRNAs selected from previous studies as regulated in the context of bone<sup>1</sup> and compared to bone turnover markers (BTMs) levels over 8 weeks in older women with and without osteoporosis. We hypothesize that miRNA varies more than BTM, and the variability is similar in women with and without osteoporosis.

## Material and methods

### Study design

We conducted a single site observational, longitudinal, case-control study of postmenopausal women within the

SHATTER study (the Low Bone Mineral Density with And without Vertebral Fractures).<sup>10,11</sup> Levels of 19 endogenous circulating miRNAs specifically selected for associations with bone based on our previous research and literature, together with 3 synthetic miRNA controls were measured in the serum of postmenopausal women with low BMD and vertebral fractures, low BMD alone and healthy control group, all group matched for age and BMI.

### Subjects

We recruited 35 postmenopausal women from 3 groups: (1) healthy controls ( $n = 12$ ); (2) patients with low BMD without any vertebral fractures ( $n = 14$ ); (3) patients with low BMD and vertebral fractures before receiving treatment ( $n = 9$ ).

BMD was measured at the LS in the posterior-anterior and lateral projections at a minimum of 2 unfractured lumbar vertebrae. Scans were acquired using the Hologic Discovery A densitometer (Hologic Inc.). Mean areal BMD ( $\text{g}/\text{cm}^2$ ) was calculated for vertebrae L1 to L4.

Vertebral fractures were identified on images from DXA-based vertebral fracture assessment (VFA) of the thoracolumbar spine using the Hologic Discovery A device. We used the algorithm-based qualitative assessment method to identify vertebral fractures and then graded their severity.<sup>12</sup> Subjects with low BMD were defined by having a spine T-score  $< -1.0$  (based on NHANES III).<sup>13</sup> All groups were matched by age and BMI (Table 1).

### Sample collection

Blood samples were collected once per week for 8 weeks at 9:00 AM after overnight fasting and avoidance of intense exercise the previous day. Serum samples were stored at  $-80^\circ\text{C}$  in the University of Sheffield Medical School Biorepository. Upon completion of the study, the samples were transferred to the South Yorkshire and North Derbyshire Musculoskeletal Biobank, which approved their use for measuring miRNA and BTMs. The South Yorkshire Research Ethics Committee approved the SHATTER study and all participants gave fully informed written consent before study enrolment.

### Bone turnover markers measurements

Serum C-terminal telopeptide of type 1 collagen (CTX) - CV 6.5%, procollagen type I N-propeptide (PINP) - CV 7.2%, osteocalcin (OC) - CV 6.3%, and bone alkaline phosphatase (BAP) - CV 3.5% were measured on the IDS-iSYS Multi-Discipline Automated Analyzer (Immunodiagnostic Systems).

### RNA extraction

The Serum/Plasma Kit (TAmiRNA GmbH) was used to perform RNA isolation. Serum samples frozen at  $-80^\circ\text{C}$  were thawed on ice and centrifuged at 12000 g for 5 min at  $4^\circ\text{C}$ . After centrifugation, precisely 200  $\mu\text{L}$  serum were mixed with 60  $\mu\text{L}$  of lysis solution and 1  $\mu\text{L}$  of RNA spike-in control by vortexing for 5 s. After incubation at room temperature for 3 min, 20  $\mu\text{L}$  protein precipitation solution was added to the homogenized sample, vigorously vortexed, and incubated for 3 min at room temperature. After centrifugation at 12 000 g for 3 min at room temperature, exactly 200  $\mu\text{L}$  of upper aqueous phase were taken and 2  $\mu\text{L}$  glycogen were added to a final concentration of 5 mg/mL. RNA was precipitated with 200  $\mu\text{L}$  of isopropanol. Samples were then transferred to columns, washed twice with wash solutions, once with 80% ethanol, and centrifuged for 5 min at 12000 g to clear out the columns from wash solution and ethanol residues. RNA

**Table 1.** Clinical description of subjects included in the study.

	Group 1 Healthy controls (n = 12)	Group 2 Low BMD without vertebral fractures (n = 14)	Group 3 Low BMD with vertebral fractures (n = 9)
Age (yr)	71.7 (7.8)	67.1 (5.8)	69.4 (6.6)
Age at menopause (yr)	47.4 (4.4)	49.4 (4.9)	52.2 (2.3)
Weight (kg)	77.2 (11.8)	67.9 (13.2)	68.1 (7.3)
Height (m)	1.61 (0.07)	1.60 (0.05)	1.61 (0.04)
BMI (kg/m <sup>2</sup> )	29.8 (3.7)	26.5 (4.5)	26.3 (2.4)
Spine BMD	1.04 (0.09)	0.83 (0.05)	0.78 (0.09)
Spine T-score	0.13 (0.73)	-1.95 (0.49)	-2.50 (0.82)
Fractures last year	0%	0%	56%
Non-vertebral fractures	0%	0%	11%
Relatives with osteoporosis	17%	36%	44%
Exercise	92%	93%	78%
Alcohol units per week	3.8 (4.4)	1.8 (2.5)	6.2 (7.3)
Smoker	17%	36%	36%

was eluted with 30  $\mu$ L of nuclease-free water, and stored at  $-80^{\circ}\text{C}$ .

### qPCR analysis

About 2  $\mu$ L of isolated RNA were used for reverse transcription to obtain cDNA, using the osteomiR chemistry Kit (TAmiRNA GmbH). Synthetic cDNA spike-in (cel-miR-39) was added as a spike-in control to monitor for enzyme inhibition during reverse transcription reaction. The reaction was incubated at  $42^{\circ}\text{C}$  for 60 min, and then heat inactivated at  $95^{\circ}\text{C}$  for 5 min. cDNA samples were stored at  $-20^{\circ}\text{C}$ . Reverse transcription quantitative PCR (RT-qPCR) analysis of 21 circulating miRNAs and 3 miRNA spike-in controls was conducted using osteomiR 384-well panels (TAmiRNA GmbH). For RT-qPCR analysis, cDNA samples were diluted 50-fold and 5  $\mu$ L were used in individual 10  $\mu$ L PCR reactions using miGreen SYBR Green master mix and LNA-enhanced miRNA primer assays together with a PCR spike-in control (TAmiRNA GmbH). RT-qPCR program was set for:  $95^{\circ}\text{C}$  for 10 min initial denaturation, 45 cycles of denaturation ( $95^{\circ}\text{C}$ , 10 s) and annealing ( $60^{\circ}\text{C}$ , 60 s), and melting curve analysis on LightCycler 480 Real-Time PCR machine (Roche). To calculate the cycle of Cq-values, the second derivative method was used.

### miRNA expression analysis

The set of 21 circulating miRNAs, including 2 hemolysis controls, was selected based on the literature and our previous studies.<sup>1</sup> We studied the following miRNAs: hsa-miR-375, hsa-miR-532-3p, hsa-miR-19b-3p, hsa-miR-152-3p, hsa-miR-23a-3p, hsa-miR-335-5p, hsa-miR-21-5p, hsa-miR-486-3p, hsa-miR-30e-5p, hsa-miR-127-3p, hsa-miR-214-3p, hsa-miR-550a-3p, hsa-miR-106b-5p, hsa-miR-133b, hsa-miR-143-3p, hsa-miR-144-3p, hsa-miR-451a, hsa-miR-29b-3p, hsa-miR-96-5p, and hsa-miR-188-5p. These miRNAs were chosen as they relate to bone metabolism; for example, has-miR-19b-3p modulates the estrogen signaling pathway, has-miR-29b-3p regulates the synthesis of extracellular matrix, has-miR-152-3p modulates ECM-receptor interaction, has-miR-152-3p modulates the signaling pathway regulating pluripotency of stem cells, and has-miR-21-5p regulates Hippo signaling pathway.<sup>11</sup> Also, we have found some of them associated with osteoporosis in our previous studies.<sup>11,14</sup>

The quality of data for each sample was tracked using synthetic miRNA spike-in controls during each step of the

workflow: RNA-Isolation (UniSp4); cDNA synthesis (cel-miR-39-3p); and RT-qPCR amplification (UniSp3). Having a quantitative quality control at each step allowed us to ensure that only samples with homogeneous purification and enzyme efficiency were considered for the final analysis. Technical variation was reduced by normalizing data against UniSp4 using the following equation:

$$\text{normalized Cq} = \text{Cq UniSp4} - \text{Cq measured miRNA}$$

The presence of hemolysis in serum samples was controlled by calculating the ratio of miR-23a-3p and miR-451a, calculated with the equation:

$$\text{hemolysis ratio} = \text{Cq hsa-miR-23a-3p} - \text{Cq hsa-miR-451a}$$

miR-23a-3p is known to be low abundant in red blood cells, while miR-451a is highly enriched and therefore changes according to the degree of hemolysis.<sup>15</sup>

Samples with hemolysis ratio higher than 7 have been excluded from further analysis.

### Statistical analysis

Statistical analysis for groups comparison was performed using GraphPad Prism (GraphPad Software) by a mixed model analysis of variance approach with statistical significance of  $P$ -value:  $P < .05$  as \*;  $P < .01$  as \*\*;  $P < .001$  as \*\*\*;  $P < .0001$  as \*\*\*\*.

To estimate intraindividual variability between weekly time-points, we have calculated the median coefficient of variation (CV). We chose to report medians as the distribution of CVs is not normally distributed.

## Results

### Subject exclusion

The set of 21 specifically selected endogenous miRNAs, including 2 miRNAs for the monitoring of hemolysis and 3 synthetic spike-in controls were measured in 8 serum samples of 35 postmenopausal women (total 280 samples).

No sample was excluded because of hemolysis. After spike-in quality control, none of the samples was removed from further analysis due to inhomogeneous reverse transcription or PCR amplification.

### Clinical characteristics

A total of 35 postmenopausal women (age, mean [SD], 69.3 [6.8] yr, weight = 71.1 [12.0] kg and height = 160.0 [5.0] cm)

**Table 2.** Vertebral fractures characteristics.

ID	Number of fractures	Location, grade, description
VF1001	1 of 1	T7, grade 1, concave
VF1007	1 of 1	T12, grade 2, wedge
VF1018	1 of 1	L1, grade 3, concave
VF1025	1 of 1	T11, grade 2, wedge
VF1029	1 of 1	T7, grade 2, biconcave
VF1031	1 of 1	T7, grade 3, wedge
VF1033	1 of 3	T7, grade 2 wedge
	2 of 3	T10, grade 2, concave
	3 of 3	T11, grade 3, wedge
VF1034	1 of 1	T10, grade 2, wedge
VF1035	1 of 1	T7, grade 3 wedge

**Table 3.** Inter-individual changes in miRNAs and BTMs levels (median CV%).

miRNA	Healthy controls	Low BMD	Vertebral fractures
hsa-miR-375	41.7%	53.8%	47.0%
hsa-miR-214-3p	80.2%	62.7%	61.4%
hsa-miR-550a-3p	29.1%	46.9%	34.6%
hsa-miR-486-3p	31.8%	52.1%	34.3%
hsa-miR-144-3p	39.7%	49.1%	43.1%
hsa-miR-532-3p	36.4%	40.4%	29.8%
hsa-miR-30e-5p	35.4%	42.3%	34.1%
hsa-miR-19b-3p	28.4%	41.0%	29.5%
hsa-miR-106b-5p	33.4%	47.2%	35.5%
hsa-miR-133b	52.7%	57.6%	49.5%
hsa-miR-152-3p	46.9%	50.2%	42.6%
hsa-miR-127-3p	71.9%	77.9%	49.0%
hsa-miR-451a	34.4%	41.0%	50.3%
hsa-miR-23a-3p	55.6%	49.3%	48.4%
hsa-miR-29b-3p	36.6%	42.5%	43.3%
hsa-miR-143-3p	50.1%	54.2%	39.1%
hsa-miR-335-5p	53.8%	57.6%	55.5%
hsa-miR-21-5p	41.0%	44.5%	38.0%
hsa-miR-96-5p	45.8%	46.8%	49.9%
hsa-miR-188-5p	43.0%	33.8%	38.5%
BTM	Healthy controls	Low BMD	Vertebral fractures
CTX	13.4%	16.8%	17.9%
PINP	13.6%	13.2%	15.6%
OC	12.7%	8.5%	11.8%
BAP	9.2%	9.8%	11.1%

were included in the analysis (Table 1). Of these, 12, 14, and 9 women were recruited to Groups 1, 2, and 3, respectively. In Group 3, 11 vertebral fractures were identified using the algorithm-based qualitative approach. Eight women had sustained one vertebral fracture and one woman had sustained 3 vertebral fractures (Table 2). All the study's vertebral fractures were most prevalent at the thoracolumbar junction (T12 to L1). We observed wedge (64%) and concave/biconcave (36%), and no compression fractures. Of all the identified fractures, 9% were categorized as grade 1, 55% as grade 2, and 36% as grade 3. None of the patients from group 3 was being treated for osteoporosis.

### miRNA expression analysis

We have measured serum levels of 21 miRNAs in the samples of 35 postmenopausal women at weekly intervals for 8 weeks.

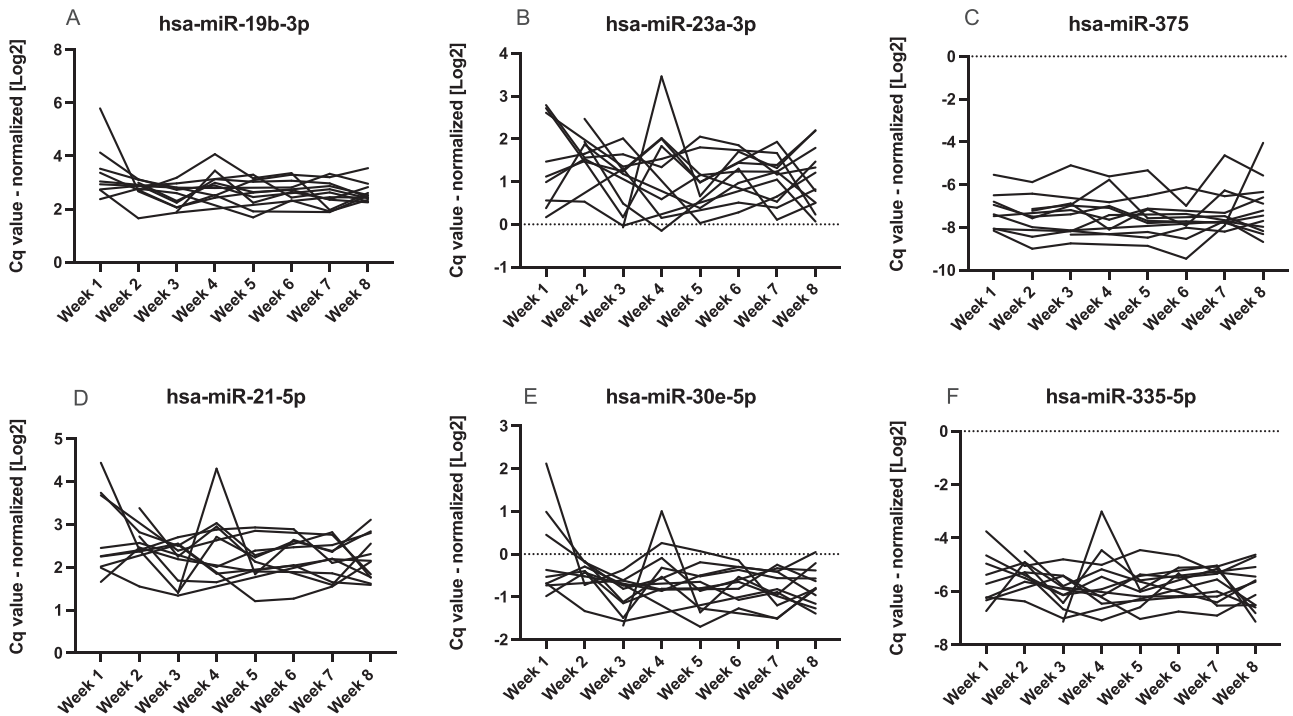
We analyzed the data using a mixed model analysis of variance approach and we found no significant changes between week-by-week time-points in any of groups.

In the healthy control group, miRNAs median intraindividual (within-subject) CV were between 28.4% (hsa-miR-19b-3p) and 80.2% (hsa-miR-214-3p); in low BMD group, 33.8% (hsa-miR-188-5p) and 77.9% (hsa-miR-127-3p); in vertebral fractures group, 29.5% (hsa-miR-19b-3p) and 61.4% (hsa-miR-214-3p) (Table 3, Figures 1–3). Combined assay CV, including RNA isolation, reverse transcription, and RT-qPCR, was 21.3% (Table 4).

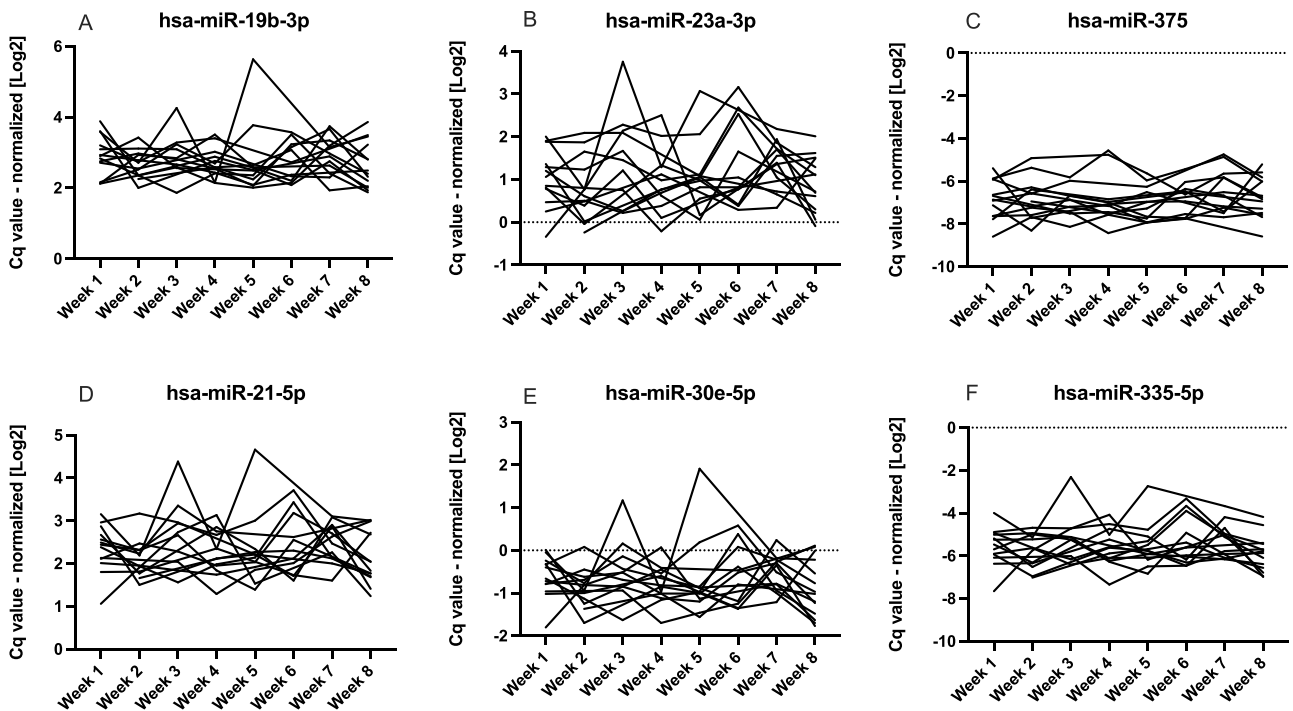
CTX intraindividual median CV was 13.4% in the healthy control group, 16.8% in the low BMD group, and 17.9% in the vertebral fractures group (Table 3, Figures 4–6), with assay CV of 6.5% (Table 4).

PINP intraindividual median CV was 13.6% in the healthy control group, 13.2% in the low BMD group, and 15.6% in the vertebral fractures group (Table 3, Figures 4–6), with assay CV of 7.2% (Table 4).

Osteocalcin intraindividual median CV was 12.7% in the healthy control group, 8.5% in the low BMD group, and 11.8% in the vertebral fractures group (Table 3, Figures 4–6), with assay CV of 6.3% (Table 4).



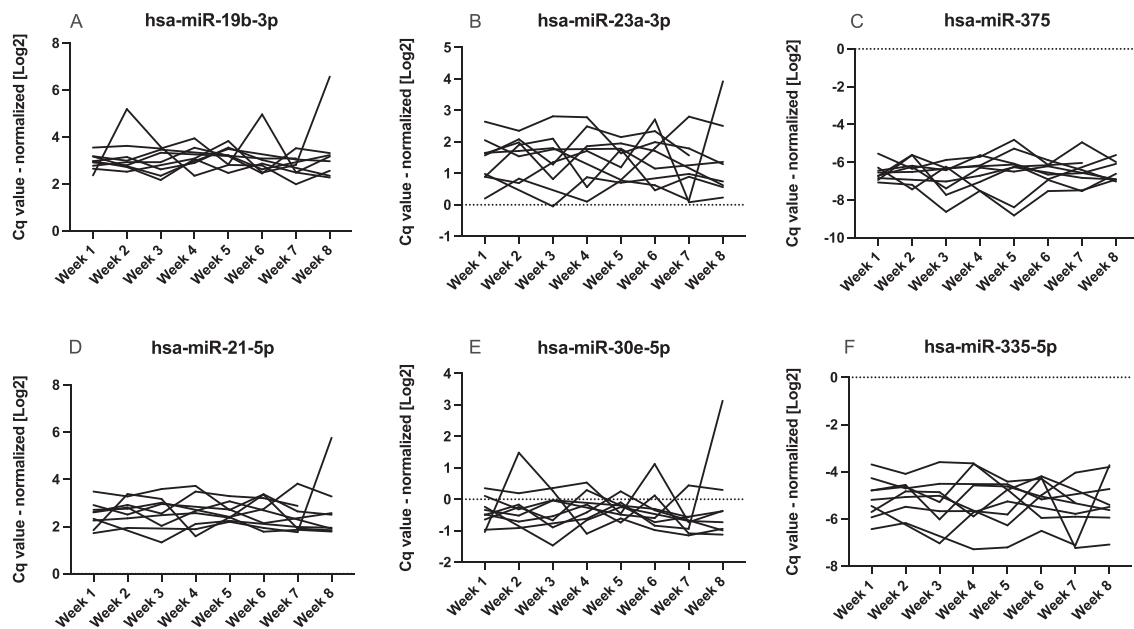
**Figure 1.** Intraindividual changes in miRNA serum levels (A-F) – healthy controls ( $n=12$ ). Serum levels of selected miRNAs were measured in the healthy control group using RT-qPCR. Cq-values were normalized to UniSP4 spike-in control. The peak seen at week 4 was from the same subject, but no cause for the peak was identified



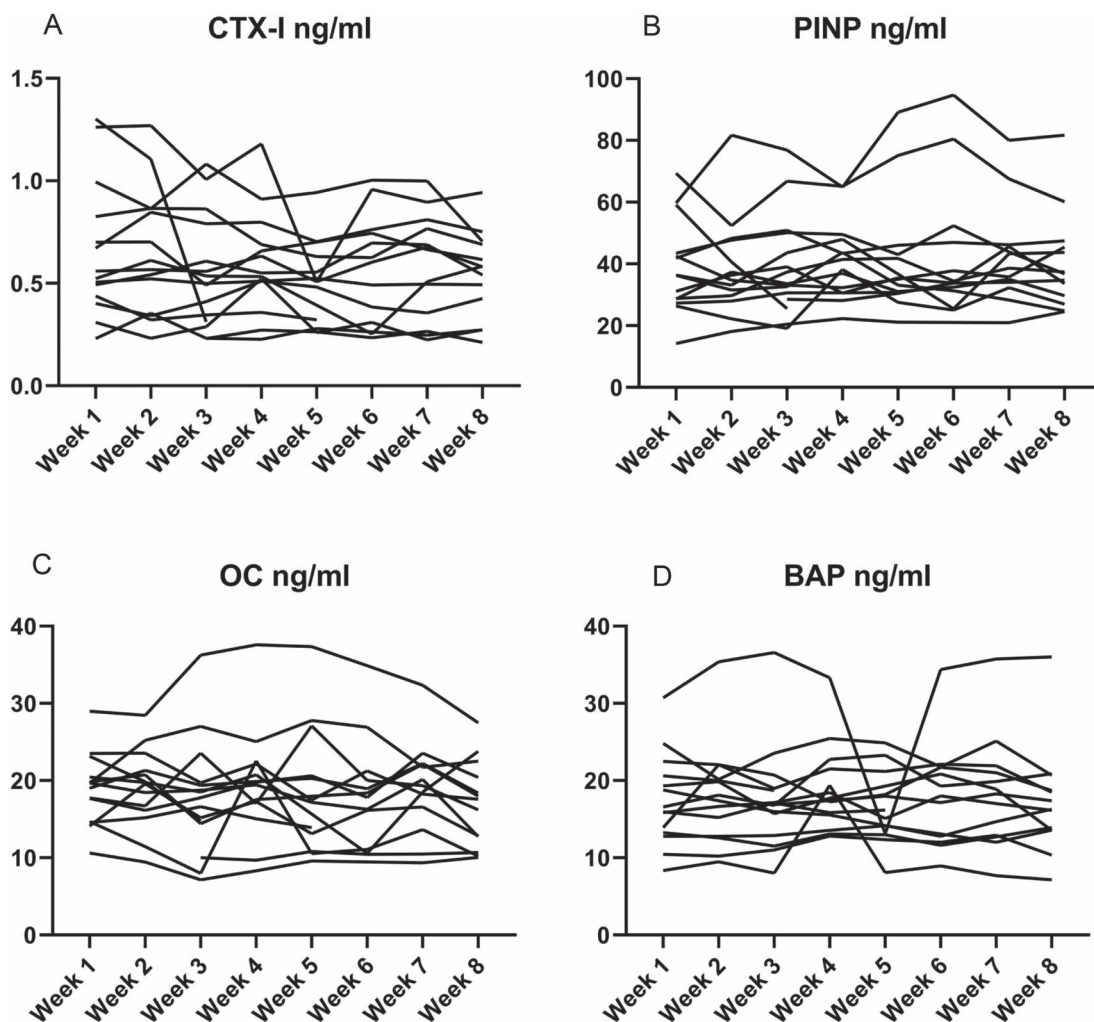
**Figure 2.** Intraindividual changes in miRNA serum levels (A-F) – low BMD ( $n=14$ ). Serum levels of selected miRNAs were measured in the low BMD group using RT-qPCR. Cq-values were normalized to UniSP4 spike-in control.

Bone alkaline phosphatase intraindividual median CV was 9.2% in the healthy control group, 9.8% in the low BMD group, and 11.1% in the vertebral fractures group (Table 3, Figures 4–6), with assay CV of 3.5% (Table 4).

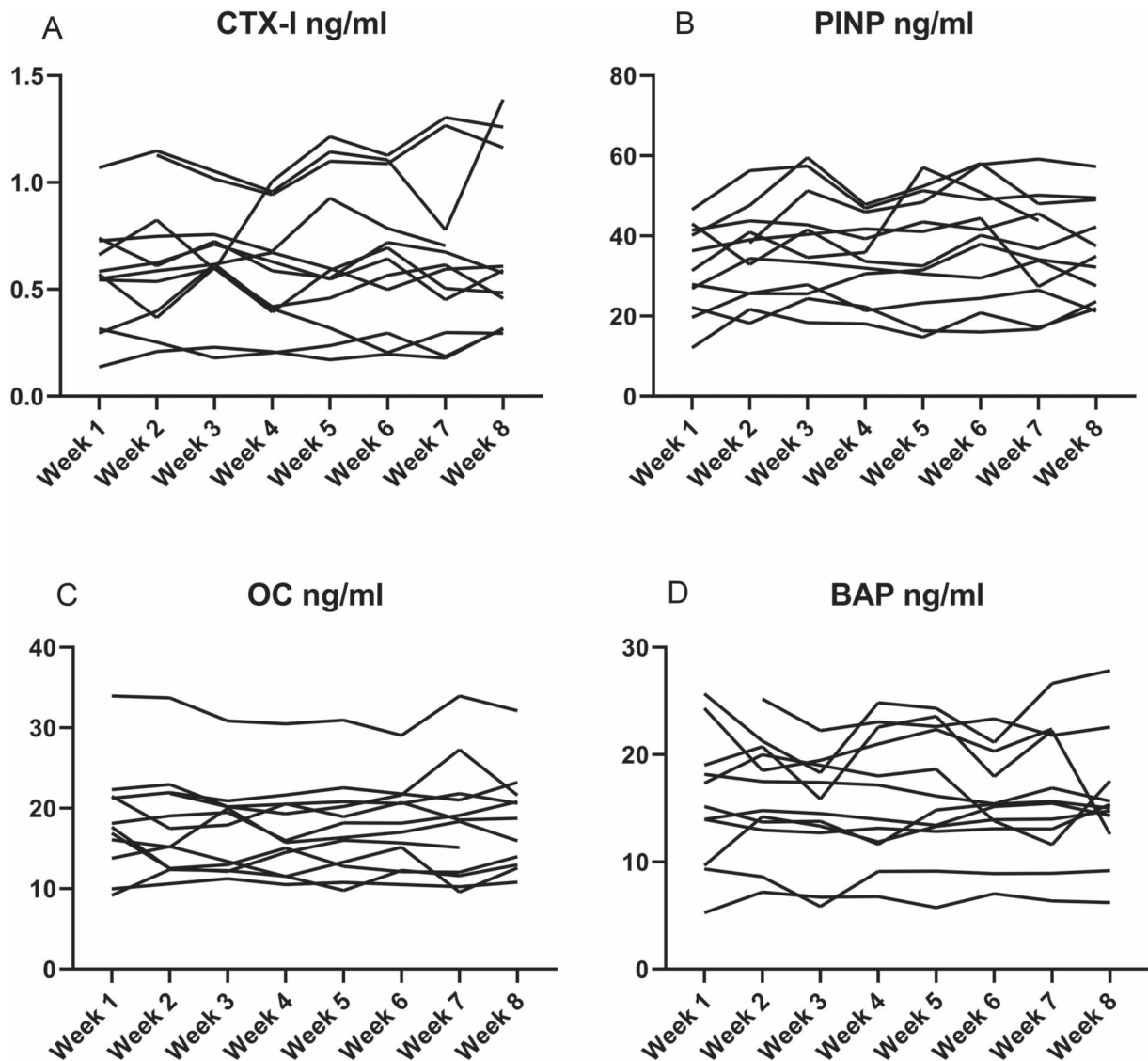
We calculated the intraindividual variability for CTX (and the key bone turnover marker) and miRNA-21-5p (the most promising miRNA based on our previous paper).<sup>11</sup> We tested the association by Spearman rank correlation, and the



**Figure 3.** Intraindividual changes in miRNA serum levels (A-F) – vertebral fractures ( $n=9$ ). Serum levels of selected miRNAs were measured in the vertebral fractures group using RT-qPCR. Cq-values were normalized to UniSP4 spike-in control.



**Figure 4.** Intraindividual changes in serum levels of bone turnover markers (A - CTX-I, B - PINP, C - OC, D - BAP) – healthy controls ( $n=12$ ). Bone turnover markers was measured in the healthy control group on the IDS-iSYS Multi-Discipline Automated Analyzer. Blood samples were collected once per week for 8 weeks at 9:00 AM after overnight fasting and avoidance of intense exercise the previous day.



**Figure 5.** Intraindividual changes in serum levels of bone turnover markers (A - CTX-I, B - PINP, C - OC, D - BAP) – low BMD ( $n=14$ ). Bone turnover markers was measured in the low BMD group on the IDS-iSYS Multi-Discipline Automated Analyzer. Blood samples were collected once per week for 8 weeks at 9:00 AM after overnight fasting and avoidance of intense exercise the previous day.

coefficient was not significant at 0.15. Thus, those subjects with the most variability in miRNA were not the same subjects most variable with CTX.

## Discussion

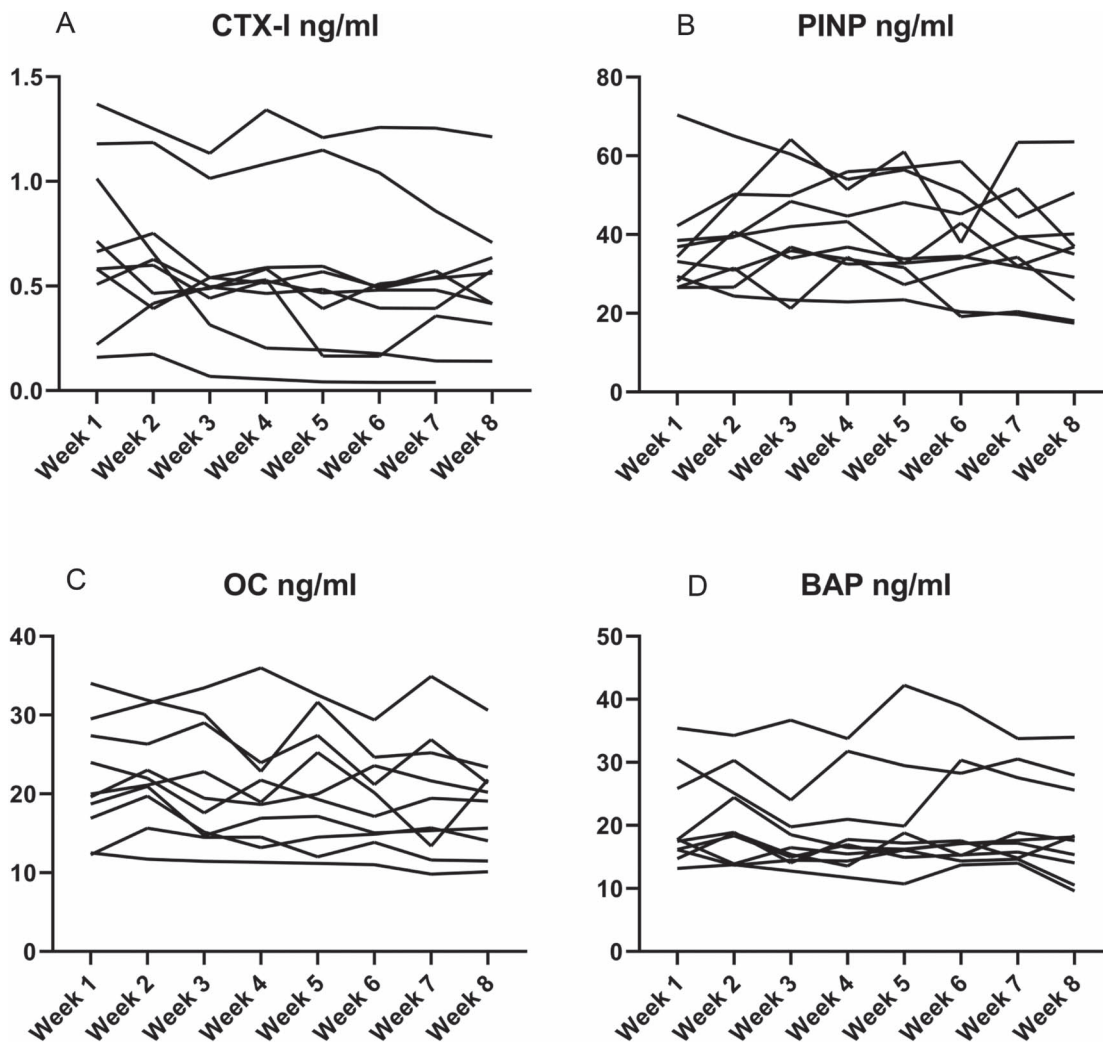
The week-to-week within-subject variability for miRNA was higher in all groups than for BTM. One cause of the higher variability was the assay CV. This was 21% for miRNA, much higher than the 3%–7% for BTM. Previous studies of variability for miRNAs gave assay variability of 6% to 69%.<sup>16</sup>

These estimates of long-term CV for miRNA are similar to those published in a study that showed a proportion of miRNAs are particularly “noisy,” more so in serum than plasma.<sup>17</sup> There is no study of the variability of miRNAs in serum over several weeks analyzed in the same way as in the present study, but some work with longer intervals, for example, annually,<sup>18</sup> (19) or in cerebrospinal fluid,<sup>19</sup> and these showed that some, but not all miRNAs show a significant degree of variability.

The next step is to reduce the assay CV for miRNAs, which may be caused by (i) manual assay handling (compared to automated BTM analysis) and (ii) multiple processing steps (RNA isolation, reverse transcription, PCR) resulting in an accumulation of variance. This could be achieved by switching to detection technologies such as antibody-based detection,<sup>20</sup> which show limited sensitivity.

The assay CVs for BTM in the present study are similar to what has been reported. In the Vasikaran<sup>21</sup> position paper, the estimates for CTX and PINP were up to 6%. In the EuBIVAS study, the CVs for CTX and PINP were 5.0% and 3.6%, a little lower than the values of 6.5% and 7.2% in the present study; however, EuBIVAS excluded 2%–4% of samples as they were outliers or affected the variance homogeneity analyses.<sup>5</sup>

The within-subject CV for BTM was similar to those published. Vasikaran<sup>21</sup> in an IOF/IFCC Position Paper summarized the data for CTX and PINP as up to 10% and 9%, and the EuBIVAS study estimated for CTX and PINP as 15.1% and 8.8%,<sup>5</sup> so the values in the present study for CTX



**Figure 6.** Intraindividual changes in serum levels of bone turnover markers (A - CTX-I, B - PINP, C - OC, D - BAP) – vertebral fractures ( $n=9$ ). Bone turnover markers were measured in the vertebral fractures group on the IDS-iSYS Multi-Discipline Automated Analyzer. Blood samples were collected once per week for 8 weeks at 9:00 AM after overnight fasting and avoidance of intense exercise the previous day.

**Table 4.** Inter-assay coefficient of variation.

miRNA assay	SD	CV%
RT-qPCR (UniSp3)	0.124	9.0%
Reverse transcription (cel-miR-39-3p)	0.212	15.9%
RNA isolation (UniSp4)	0.279	21.3%
BTM assay	CV%	
CTX	6.5%	
PINP	7.2%	
OC	6.3%	
Bone ALP	3.5%	

To estimate assay variability, we measured the levels of synthetic spike-in controls over the course of 8 weeks in each sample. We calculated a median coefficient of variation (CV%) using spike-in controls added during each step of the workflow. UniSp4 was added at the beginning of the RNA isolation protocol and was present in samples throughout the whole workflow. Cel-miR-39-3p was added to monitor for enzyme inhibition during reverse transcription reaction. UniSp3 was added before the RT-qPCR reaction to compare reaction efficiency between each sample.

of 13 to 18, and for PINP of 13% to 16% are similar, if not identical.

The sources of variability of bone turnover markers have been studied thoroughly.<sup>22</sup> Thus, for CTX, there can be

variability within an individual due to the type of sample (serum or plasma), due to a recent meal (lower values), sampling in the afternoon rather than the morning (lower values), intense exercise (higher values), and the presence of diseases or the use of drugs. To reduce these sources of variability, it is usually recommended that CTX is taken from a fasting subject in the morning and that intense exercise is avoided the previous day, as was done in this study. The same determinants of variability for BTM are likely present for miRNAs: sample processing is a major source of variability evidenced by the large differences in miRNA profiles between serum and plasma,<sup>23</sup> and the impact of plasma processing conditions.<sup>24</sup> Furthermore, circadian rhythm,<sup>24</sup> feeding effect, and day-to-day variability need further study.

The effects of osteoporosis treatments on BTMs are well described. The most standard treatments for osteoporosis are bisphosphonates. The percent decrease in BTMs is much greater than the within-subject variability in most subjects with postmenopausal osteoporosis and the ratio is referred to as the signal-to-noise ratio.<sup>4</sup> The within-subject variability can be used to calculate the least significant change to identify responders.<sup>25</sup> More than 80% of patients can be considered responders after 12 weeks of treatment with oral bisphosphonates.<sup>6</sup>



There is only preliminary information about osteoporosis therapy's effects on miRNAs, due to which the magnitude of the response has yet to be discovered, for example, the effect of bisphosphonate therapy on miRNA in man. There is some evidence for changes in miR-33-3p and mir-133a in response to teriparatide, as well as increases in miR-26b-5p and miR-454-3p of 100% to 200%,<sup>26</sup> although no change was reported by others.<sup>27</sup> The need for more consistency in these studies is likely explained by the high variability in miRNA measurements and points to the need to have studies that are sufficiently powered. These clinical studies were conducted in women with low BMD with or without vertebral fracture, so the information included in the present paper is relevant to these. Once we have this information, we can evaluate the signal-to-noise ratio for miRNA monitoring of osteoporosis treatment.

In conclusion, circulating miRNAs measured in serum have a higher weekly intraindividual variability than bone turnover markers due partly to a higher assay CV. Strategies are required to reduce this variability. The high variability needs to be considered when designing future studies that include miRNAs.

## Acknowledgments

We are grateful to Dr Margaret Paggiosi for reading the VFA images and measuring BMD. The reagents for the bone turnover markers were provided to us by Immunodiagnosics Systems. Dr Debono was funded by a Fellowship from the National Institute of Health Research and Patryk Zarecki by a collaborative studentship from the University of Sheffield, supported by TAmiRNA GmbH. We appreciate South Yorkshire and North Derbyshire Biobank for releasing the samples for miRNA and BTM measurement.

## Author contributions

Patryk Zarecki (Conceptualization, Data curation, Formal analysis, Methodology, Project administration, Writing—original draft, Writing—review & editing), Fatma Gossiel (Methodology, Resources, Validation, Writing—review & editing), Johannes Grillari (Conceptualization, Investigation, Resources, Methodology, Project administration, Writing—review & editing), Miguel Debono (Investigation, Project administration, Writing—review & editing), Matthias Hackl (Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Formal analysis, Writing—review & editing), and Richard Eastell (Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing—original draft, Writing—review & editing)

## Funding

The University of Sheffield and TamiRNA GmbH funded the study through a PhD studentship.

## Conflicts of interest

M.H. and J.G. are co-founders of TAmiRNA. TAmiRNA employs M.H. J.G. is a scientific advisor to TAmiRNA. M.H. and J.G. hold patents related to the application of circulating microRNAs as biomarkers for the diagnosis of bone disorders. R.E. receives grant funding from TAmiRNA and Immunodiagnosics Systems. The remaining authors have nothing to disclose.

## Data availability

Data are available from the authors on reasonable request as part of a collaboration project.

## References

1. Grillari J, Makitie RE, Kocijan R, et al. Circulating miRNAs in bone health and disease. *Bone*. 2021;145:115787. <https://doi.org/10.1016/j.bone.2020.115787>.
2. Griffiths-Jones S. The microRNA registry. *Nucleic Acids Res*. 2004; 32(90001):109D–1111D. <https://doi.org/10.1093/nar/gkh023>
3. Hackl M, Heilmeier U, Weinel S, Grillari J. Circulating microRNAs as novel biomarkers for bone diseases - complex signatures for multifactorial diseases? *Mol Cell Endocrinol*. 2016;432:83–95. <https://doi.org/10.1016/j.mce.2015.10.015>
4. Machado A, Hannon R, Eastell R. Monitoring alendronate therapy for osteoporosis. *J Bone Miner Res*. 1999;14(4):602–608. <https://doi.org/10.1359/jbmr.1999.14.4.602>
5. Cavalier E, Lukas P, Bottani M, et al. European biological variation study (EuBIVAS): within- and between-subject biological variation estimates of beta-isomerized C-terminal telopeptide of type I collagen (beta-CTX), N-terminal propeptide of type I collagen (PINP), osteocalcin, intact fibroblast growth factor 23 and uncarboxylated-unphosphorylated matrix-Gla protein—a cooperation between the EFLM Working Group on Biological Variation and the International Osteoporosis Foundation-International Federation of Clinical Chemistry Committee on Bone Metabolism. *Osteoporos Int*. 2020;31(8):1461–1470. <https://doi.org/10.1007/s00198-020-05362-8>
6. Naylor KE, Jacques RM, Paggiosi M, et al. Response of bone turnover markers to three oral bisphosphonate therapies in postmenopausal osteoporosis: the TRIO study. *Osteoporos Int*. 2016;27(1):21–31. <https://doi.org/10.1007/s00198-015-3145-7>
7. Diez-Perez A, Naylor KE, Abrahamsen B, et al. International Osteoporosis Foundation and European Calcified Tissue Society Working Group. Recommendations for the screening of adherence to oral bisphosphonates. *Osteoporos Int*. 2017;28(3):767–774. <https://doi.org/10.1007/s00198-017-3906-6>
8. Vogt J, Sheinson D, Katavolos P, et al. Variance component analysis of circulating miR-122 in serum from healthy human volunteers. *PLoS One*. 2019;14(7):e0220406. <https://doi.org/10.1371/journal.pone.0220406>
9. Keller A, Rounge T, Backes C, et al. Sources to variability in circulating human miRNA signatures. *RNA Biol*. 2017; 14(12):1791–1798. <https://doi.org/10.1080/15476286.2017.1367888>
10. Paggiosi MA, Debono M, Walsh JS, Peel NFA, Eastell R. Quantitative computed tomography discriminates between postmenopausal women with low spine bone mineral density with vertebral fractures and those with low spine bone mineral density only: the SHATTER study. *Osteoporos Int*. 2020;31(4):667–675. <https://doi.org/10.1007/s00198-020-05317-z>
11. Zarecki P, Hackl M, Grillari J, Debono M, Eastell R. Serum microRNAs as novel biomarkers for osteoporotic vertebral fractures. *Bone*. 2020;130:115105. <https://doi.org/10.1016/j.bone.2019.115105>
12. Jiang G, Eastell R, Barrington NA, Ferrar L. Comparison of methods for the visual identification of prevalent vertebral fracture in osteoporosis. *Osteoporos Int*. 2004;15(11):887–896. <https://doi.org/10.1007/s00198-004-1626-1>
13. Looker AC, Orwoll ES, Johnston CC Jr, et al. Prevalence of low femoral bone density in older U.S. adults from NHANES III. *J Bone Miner Res*. 1997;12(11):1761–1768. <https://doi.org/10.1359/jbmr.1997.12.11.1761>
14. Kerschhan-Schindl K, Hackl M, Boschitsch E, et al. Diagnostic performance of a panel of miRNAs (OsteomiR) for osteoporosis in a cohort of postmenopausal women. *Calcif Tissue Int*. 2021;108(6): 725–737. [10.1007/s00223-020-00802-3](https://doi.org/10.1007/s00223-020-00802-3)

15. Blondal T, Jensby Nielsen S, Baker A, et al. Assessing sample and miRNA profile quality in serum and plasma or other biofluids. *Methods*. 2013;59(1):S1–S6. <https://doi.org/10.1016/j.ymeth.2012.09.015>
16. Brunet-Vega A, Pericay C, Quilez ME, Ramirez-Lazaro MJ, Calvet X, Lario S. Variability in microRNA recovery from plasma: comparison of five commercial kits. *Anal Biochem*. 2015;488:28–35. <https://doi.org/10.1016/j.ab.2015.07.018>
17. Ammerlaan W, Betsou F. Intraindividual temporal miRNA variability in serum, plasma, and white blood cell subpopulations. *Biopreserv Biobank*. 2016;14(5):390–397. <https://doi.org/10.1089/bio.2015.0125>
18. Orr C, Myers R, Li B, et al. Longitudinal analysis of serum microRNAs as predictors of cirrhosis regression during treatment of hepatitis B virus infection. *Liver Int*. 2020;40(7):1693–1700. <https://doi.org/10.1111/liv.14474>
19. Yoon H, Belmonte KC, Kasten T, Bateman R, Kim J. Intra- and inter-individual variability of microRNA levels in human cerebrospinal fluid: critical implications for biomarker discovery. *Sci Rep*. 2017;7(1):12720. <https://doi.org/10.1038/s41598-017-13031-w>
20. Kappel A, Backes C, Huang Y, et al. MicroRNA in vitro diagnostics using immunoassay analyzers. *Clin Chem*. 2015;61(4):600–607. <https://doi.org/10.1373/clinchem.2014.232165>. PubMed PMID: 25617425
21. Vasikaran S, Eastell R, Bruyere O, et al. Markers of bone turnover for the prediction of fracture risk and monitoring of osteoporosis treatment: a need for international reference standards. *Osteoporos Int*. 2011;22(2):391–420. <https://doi.org/10.1007/s00198-010-1501-1>. PubMed PMID: 21184054.
22. Szulc P, Naylor K, Hoyle NR, Eastell R, Leary ET, National Bone Health Alliance Bone Turnover Marker P. Use of CTX-I and PINP as bone turnover markers: National Bone Health Alliance recommendations to standardize sample handling and patient preparation to reduce pre-analytical variability. *Osteoporos Int*. 2017;28(9):2541–2556. <https://doi.org/10.1007/s00198-017-4082-4>
23. Wang K, Yuan Y, Cho JH, McClarty S, Baxter D, Galas DJ. Comparing the MicroRNA spectrum between serum and plasma. *PLoS One*. 2012;7(7):e41561. <https://doi.org/10.1371/journal.pone.0041561>
24. Mussbacher M, Krammer TL, Heber S, et al. Impact of anticoagulation and sample processing on the quantification of human blood-derived microRNA signatures. *Cells*. 2020;9(8):1915. <https://doi.org/10.3390/cells9081915>
25. Hannon R, Blumsohn A, Naylor K, Eastell R. Response of biochemical markers of bone turnover to hormone replacement therapy: impact of biological variability. *J Bone Miner Res*. 1998;13(7):1124–1133. <https://doi.org/10.1359/jbmr.1998.13.7.1124>
26. Messner Z, Carro Vazquez D, 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. <https://doi.org/10.1210/clinem/dgac667>
27. 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>