ORIGINAL ARTICLE

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Lack of Association Between Select Circulating miRNAs and Bone Mass, Turnover, and Fractures: Data From the OFELY Cohort

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ABSTRACT

Postmenopausal osteoporosis is characterized by the occurrence of fragility fracture with an increase in morbidity and mortality. Recently, microRNAs (miRNAs) have raised interest as regulators of translational repression, mediating a number of key processes, including bone tissue in both physiological and diseased states. The aim of this study was to examine the serum levels of 32 preselected miRNAs with reported function in bone and their association with osteoporotic fracture. We performed cross-sectional and longitudinal analyses from the OFELY Cohort. Serum levels of the miRNAs were quantified by qRT-PCR in 682 women: 99 premenopausal and 583 postmenopausal women, with 1 and 122 women with prevalent fragility fractures in each group, respectively. We have collected clinical variables (such as age, prevalent, and incident fractures), bone turnover markers (BTMs), BMD by dual X-ray absorptiometry, and bone microarchitecture with HRpQCT. We observed a number of miRNAs to be associated with fragility fractures (prevalent or incident), BTMs, BMD, and microarchitecture. This effect, however, was negated after age adjustment. This may be because age was also strongly associated with the serum levels of the 32 miRNAs (correlation coefficient up to 0.49), confirming previous findings. In conclusion, in a well-characterized prospective cohort with a sizeable sample size, we found no evidence that these 32 preselected miRNAs were not associated with BTMs, BMD, microarchitecture, and or fragility fractures. © 2019 American Society for Bone and Mineral Research.

KEY WORDS: BONE QCT/MICROCT; DXA; OSTEOPOROSIS; DISEASES AND DISORDERS OF/RELATED TO BONE; AGING

Introduction

Osteoporosis is characterized by low bone mass and a deterioration of bone structure. This condition leads to bone fragility, increasing the likelihood of fracture. DXA is the mainstay of bone fragility assessment. Prospective studies have demonstrated the association between low BMD and the risk of fractures in women[1] and men.[2] However, the diagnostic threshold of the *T*-score at −2.5 SD identifies only half of the women and 20% of men who will sustain a fragility fracture.[3–5] Numerous studies have addressed this limitation by looking for biological and imaging markers that may improve the identification of individuals at risk. For example, several microarchitectural parameters can be measured noninvasively by methods such as HRpQCT. HRpQCT has been reported to be predictive of incident fracture, independently of areal BMD.[6–8] However, this method is not yet accessible in clinical practice; hence, alternate measures of bone fracture risk are needed.

MicroRNAs (miRNAs) are short, noncoding single-stranded RNAs that are involved in posttranscriptional regulation of gene expression, leading to repression of protein translation.[9] MiRNAs are major regulators of cell differentiation, proliferation, and maturation. They have been shown to mediate bone homeostasis and are involved in osteoblastogenesis and osteoclastogenesis regulation.[10–14] A recent study has shown in vivo injection of anti-miR-214-induced improvement of bone microarchitecture and bone mass in mice.[15] Other reports also suggest an association between the serum levels of some miRNAs with human osteoporosis.[16–20] Seeliger and colleagues reported miR-21, miR-23a, miR-24, miR-25, miR-100, and miR-125b to be significantly higher in the serum of patients with osteoporosis than healthy individuals.[16]

Previously, miR-133a-3p has been demonstrated to play a role in bone, and was increased after menopause in ovariectomized mice and in postmenopausal women (plasma and circulated monocytes).[21–23] This effect may be mediated through miR-133a-3p targeting Runx2 to decrease osteoblastogenesis.[24]

Moreover, miRNAs are released in the biofluids from various tissues, including bone, and can be measured from blood samples. In addition, it has been shown that circulating miRNAs are particularly stable under extreme conditions (boiling, low or high pH, extended storage, freeze–thaw cycles).[25,26] This stability may be partly because of their short length, miRNA integration within microparticles such as exosomes,[27] or their

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association with protein[28] or lipoprotein complexes.[29] Considering all these features, circulating miRNAs emerge as promising biomarkers.

Therefore, the aim of this study was first to examine the association of the serum level of 32 miRNAs with bone turnover, bone density, and volumetric bone mineral density (vBMD). The baseline serum level of the miRNAs were quantified in a cross-sectional analysis of the OFELY Cohort and statistically investigated in relation to baseline bone turnover marker (BTM), DXA, and HRpQCT values, and prevalent fracture. We also wanted to determine whether the potential relationship between bone variables and microRNAs differed between pre- and postmenopausal women, which would be suggestive of an influence of estrogen. Second, the association between miRNAs and incident fragility fractures in postmenopausal women from the same OFELY Cohort was analyzed in a prospective analysis.

Materials and Methods

Subjects

All subjects were participants of the OFELY (Os des Femmes de Lyon) Cohort, a prospective monocentric cohort established to study the determinants of bone loss in women.[30] The women were recruited from a French health care insurer (Mutuelle Générale de l'Education Nationale) between February 1992 and December 1993. This study initially included 1039 women aged 31 to 89 years (Fig. 1). All subjects gave their informed consent and the protocol was approved by the local ethics committee.

The 14th year of follow-up was selected as the baseline for the cross-sectional analysis of the study because it was the first year with a HRpQCT assessment. Between 2005 and 2007, 682 women came for their 14th visit (age 44 to 95 years).

Menopause was defined by an absence of menstruation for at least one year at baseline.

Fracture assessment

Fractures were defined by the occurrence of a fragility fracture based on medical history, and ascertained with medical records or spine X-rays for vertebral fractures. Only fragility fractures resulting from low trauma (ie, those occurring from standing height or less) were taken into account and fractures of the head, toes, and fingers were excluded.

Prevalent fractures that occurred before the inclusion in the study (pre-1992 to 1993), but after age 40 years, and all fractures occurring after the inclusion and before the 14th follow-up were recorded. Incident nonvertebral and clinical vertebral fractures were annually registered between the observation period and the last follow-up visit for that study (2013 to 2014) through questionnaires and were all confirmed by radiographs or surgical reports.

Bone mineral density measurements

Areal aBMD was measured by DXA with a Hologic QDR 4500 device (Hologic, Waltham, MA, USA) at the total hip, femoral neck, and lumbar spine (L1 to L4). DXA reproducibility was assessed in vivo, with a coefficient of variation of 0.9% for the lumbar spine and 1% for the total hip region. A control phantom scan was performed on a daily basis and all measurements were made by the same experienced technician.

HRpQCT

Microarchitectural parameters were measured with HRpQCT using the XtremeCT (ScancoMedical AG, Brüttisellen, Switzerland) at the nondominant distal radius and tibia. One hundred ten parallel and contiguous slices were obtained with an isotropic size voxel of 82 μm. The in vivo reproducibility of this device is 0.7% to 1.5% for total, trabecular, and cortical BMD.[31]

Sample collection and handling

Blood samples were collected between 8:00 a.m. and 9:30 a.m. after an overnight fast. Serums were aliquoted in tubes of 1 mL and frozen at −80°C until assayed. Samples from the 14th follow-up (without previous freeze–thaw cycle) were thawed on ice and centrifuged at 3000*g* for 5 min. Hemolysis was determined by macroscopic analysis and classified as absent, low, moderate, and high.[32]

Biochemistry

The BTMs (P1NP, CTX-1, and osteocalcin) were obtained in a series of measurements, by automated tests for each parameter, at the end of the 14th follow-up (elecsys P1NP, elecsys β-crosslaps, elecsys N-MID osteocalcin, respectively; Roche Diagnostics, Meylan, France). Serum bone alkaline phosphatase (BAP) was measured by ELISA (Metra BAP EIA Kit; Quidel Corp,

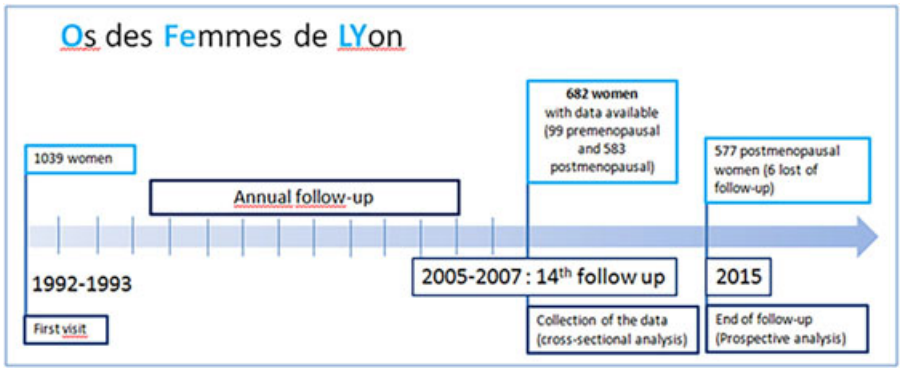

Fig. 1. Flowchart of the cohort.

San Diego, CA, USA). Intra- and interassay variations were <8% for all markers.

miRNA quantification

Preselection of the miRNAs

Literature searches were performed between September and December 2014 using PubMed (https://www.ncbi.nlm.nih.gov/m/pubmed/) to identify candidate miRNAs for analysis in the OFELY Cohort. The key words used for the researches were "microRNA," "osteoporosis," and "bone." The selected articles were those highlighting an association between osteoporosis, fracture, bone turnover, and miRNAs. The final choice was made based on the strength of the associations described in the different reviewed articles. Specifically, we chose to further study those microRNAs that were significantly associated with prevalent fracture (there was no study finding an association with incident fracture in healthy postmenopausal women), BMD, and bone turnover. After critical assessment, 32 miRNAs were selected based on their reported function in the osteoporosis process, both in basic (in vitro and in vivo) and/or clinical research. A complete list of the 32 miRNAs and their sequences are provided in Supplementary Table S1. Table 1 features a summarization of the main articles used to make the selection, and also includes the major articles published after the selection.

Small RNA isolation from serum

All the next steps were performed by the same trained operator (EF) in 2015. Small RNAs were extracted from 200 μL of serum using the miRCURY Biofluids RNA Isolation Kit (Exiqon, Vedbaek, Denmark) according to the manufacturer's protocol, except for the addition of 1 μL of glycogen and 1 μL of a synthetic spike-in control RNA (UniSp6; RNA Spike-In Template UniSp6, Ref 203301-02, Exiqon) to lysis buffer (to improve extraction yield and as internal control, respectively). Samples were treated with an on-column rDNase digestion (supplied) before elution in 40 μL of RNase-free water and stored at −80°C.

miRNA quantification by qRT-PCR

miRNA levels in the serum were quantified on a custom-designed Pick-&-Mix microRNA PCR panel using locked nucleic acid (LNA) probes (miRCURY LNA Universal RT; Exiqon,) following reverse transcription of 4 μL with the miRCURY LNA Universal RT microRNA PCR (Exiqon) according to the manufacturer's protocol. The resulting cDNA product was diluted 1:20, of which 4.5 μL was added to 5.5 μL of ExiLENT SYBR Green Master Mix (Exiqon). miRNAs were assayed in 96-well plates with the cycling conditions: 10 min at 95°C and 50 cycles of 95°C for 10 s and 60°C for 60 s, followed by a melt curve analysis (Mastercycler Realplex; Eppendorf, Hamburg, Germany). We attributed a value of 50 to the cycle threshold (C_T) of the miRNA; expression levels were below the detection limit. Data on reproducibility are provided in the supplementary data.

Relative quantification was calculated by the ΔΔCt method, relative to an exogenous spike-in control, UniSP6 (ΔCt), and the mean of each miRNA (irrespective of menopausal status) (ΔΔCt). Data on reproducibility are available in Supplementary Fig. S1.

Statistical analyses

The baseline characteristics of the women were presented as mean [min–max] for continuous variables and number (percentage) of patients for categorical variables. A cross-sectional analysis was performed using the Student *t* test to assess the cohort for pre- and postmenopausal women. The analysis of the distribution between the different groups of women used the Wilcoxon test; logistic regression was applied for age adjuestment because none of the miRNAs had a normal distribution.

The Wilcoxon test was used to examine the significance of the difference between the serum miRNA levels in women with and without incident fracture. Again, significant results were then adjusted for age with logistic regression.

All the analyses were performed with the STATA/IC software (version 12 for Windows 64-bit x86-64; StataCorp, College Station, TX, USA). A p value of <0.05 was considered significant.

Results

Data on the power calculation are available in Supplementary Table S2.

Cohort characteristics

Characteristics of the cross-sectional study are summarized in Table 2. Data were available for 682 women. Serum from these women was examined: 99 women were premenopausal and 583 were postmenopausal. Only one woman had a prevalent fracture in the premenopausal group (metatarsal fracture), whereas 122 patients had at least one fragility fracture in the postmenopausal group: 40 with vertebral fracture(s) (19 grade 1 according to Genant score, 14 grade 2 and 7 grade 3), 2 with fracture of the hip, 40 with wrist fracture, 18 with fracture of the lower end of the tibia, 6 with fracture of the proximal humerus, and 1 pelvic fracture). Among the 122 participants with prevalent fracture observed in the postmenopausal group, 5 fractures occurred fewer than 6 months before the baseline time point.

Osteoporotic-related drugs were used in 171 postmenopausal women, including hormone replacement therapy ($n=58$), bisphosphonates ($n=81$), SERM (selected estrogen receptor modulator, $n=16$), tibolone ($n=10$), and in 2 premenopausal women, including bisphosphonates ($n=1$) and SERM ($n=1$). Bone loss-inducing therapy was used in 13 postmenopausal women including aromatase inhibitors ($n=11$) and corticosteroids ($n=2$). A combination of treatment was observed in 7 women.

The mean estimated glomerular filtration rate by the CKD EPI equation was 84 mL/min/1.73 m^2 (minimum 24 mL/min/1.73 m^2, maximum 115 mL/min/1.73 m^2). None of the patients of our cohort had terminal renal failure (stage 5; CKD EPI < 15 mL/min/1.73 m^2) and only 2 women (from the postmenopausal group) had stage 4 renal failure (CKD EPI between 15 and 30 mL/min/1.73 m^2). In addition, we counted 5 patients with treated hyperthyroidisms, no hyperparathyroidism, 3 rheumatoid arthritis patients, and 18 patients with diabetes (3% of the women). These low frequencies did not allow us to study the influence of those diseases.

In this study, postmenopausal women were shorter by an average of 3 cm. Body weight was not significantly different between pre- and postmenopausal women. BMI tended to be higher in postmenopausal women, which was linked to the shorter height.

As expected, the BMD values were lower in postmenopausal women in comparison with premenopausal women (13% lower for the neck BMD, 12% for the total hip-area BMD, 11% for the

Table 1. Main Literature Support for the Selection of the 32 miRNAs and Main Target Genes for Each miRNA

Selected miRNAs	Target genes	Description (can be discordant) and main literature support
hsa-miR-133a-3p	Runx2	Increased in ovariectomized mice[21] and in postmenopausal women.[22,23] Targets Runx2.[24]
hsa-miR-20a-5p	PPARγ, Bambi, Crim1	Enhances osteoblastogenesis in human mesenchymal stem cells,[58] and decreased during osteoclastogenesis.[59]
hsa-miR-25-3p	?	Increased in bone sample of osteoporosis patients.[16]
hsa-miR-100-5p	BMPR2	Increased in bone and serum sample of osteoporosis patients.[16,34]
hsa-miR-133b	?	Decreased in subjects with fracture,[17] increased in ovariectomized mice.[21]
hsa-miR-214-3p	ATF4	Enhances osteoclastogenesis in vitro and in vivo in mice,[60] and inhibits bone formation in bone sample from aged patients with fractures by targeting ATF4.[61]
hsa-miR-26a-5p	Smad1, CDK6, HDAC4	Inhibits late osteoblastogenesis stage by targeting Smad1 in human adipose tissue-derived stem cells,[62] and increased during osteogenic differentiation of unrestricted somatic stem cells from human cord blood.[63]
hsa-miR-103a-3p	Runx2, Cav1.2	Inhibits osteoblastic proliferation as a mechanosensitive miRNA by targeting Runx2 in vitro and in vivo.[64,65]
hsa-miR-145-5p	Cbfb, Sox9	Inhibits osteoblastogenesis in vitro,[66] and inhibits early stage of chondrogenic differentiation by targeting Sox9.[67]
hsa-miR-21-5p	Spry1, PDCD4	Increased in bone and serum sample of osteoporosis patients,[16] and in serum of osteoporosis women with fracture[33] Decreased in serum of osteoporosis women with vertebral fracture,[20] and in plasma of postmenopausal women with low bone mass.[22]
hsa-miR-29a-3p	HDAC4	Decreased in serum of patients with low bone mass,[20] and enhances osteoblastic differentiation and mineral acquisition under glucocorticoids treatment.[68,69]
hsa-miR-106a-5p	BMP2	Inhibits osteoblastic differentiation by enhancing adipogenic differentiation,[70] and is decreased during osteoblastogenesis in mesenchymal stem cells.[71]
hsa-miR-146a-5p	FGF2, TRAF6, EGFR	Polymorphisms of FGF2 binding sites and miR–146a are associated with variations of BMD,[72,73] and inhibit osteoclastogenesis in vitro.[74]
hsa-miR-221-5p	Runx2	Decreased during osteoblast differentiation in vitro in exosomes of human mesenchymal stem cells[75] and in human bone cells.[76]
hsa-miR-29b-3p	Cdc42, Srgap2	Decreased in vivo in serum of patients with fractures,[19] in serum of aged patients[77] Enhances osteoclastogenesis in vitro.[78]
hsa-miR-122-5p	?	Increased in serum of osteoporosis patients, and in patients with fractures.[16,33,34]
hsa-miR-148a-3p	MAFβ	Increased in serum[16] and plasma of osteoporosis patients.[79]
hsa-miR-222-3p	Smad5, Runx2	Increased in serum of aged patients,[77] and inhibits osteoblastogenesis in vitro in human mesenchymal stem cells.[80]
hsa-miR-338-3p	Runx2	Inhibits osteoblastic differentiation in vitro.[81,82]
hsa-miR-124-3p	Dlx2, Dlx3, Dlx5	Increased in the serum of osteoporosis patients,[16,20] and inhibits osteoblastic differentiation in vitro and in vivo in mice.[83]
hsa-miR-155-5p	SOCS1, MITF	Decreased in serum of diabetic women with fracture,[18] and inhibits osteoclastogenesis and osteoblastogenesis in vitro.[12,84]
hsa-miR-223-5p	NF1–A	Influences osteoclastogenesis, with variable effects depending on the concentration of the miRNA.[11,85]
hsa-miR-34a-5p	JAG1, Tgif2	Inhibits in vitro osteoblastogenesis by targeting JAG1,[86] and osteoclastogenesis by targeting Tgif2.[87]
hsa-miR-125b-5p	Erbβ2	Increased in bone sample[16] and serum of osteoporosis patients,[16,33,34] and inhibits osteoblastic differentiation in vitro.[35]
hsa-miR-17-5p	BMP2	Inhibits osteoblastic differentiation by enhancing adipogenic differentiation.[70]
hsa-miR-23a-3p	Runx2	Increased in bone sample and serum of osteoporosis patients,[16] while decreased in serum of patients with low bone mass.[20]
hsa-miR-503-5p	RANK	Decreased in peripheral blood mononuclear cells of osteoporosis postmenopausal women, and inhibits osteoclastogenesis by targeting RANK.[88]
hsa-miR-127-3p	Runx2	Decreased in ovariectomized mice and enhances osteoclastogenesis in vitro,[21] enhances chondrogenic differentiation at the expense of osteoblastic differentiation.[89]
hsa-miR-204-5p	Runx2	Decreased in ovariectomized mice,[21] and inhibits osteoblastic differentiation in vitro.[81,90]
hsa-miR-24-3p	Runx2	Increased in bone sample[16] and serum of osteoporosis patients.[16,34]
hsa-miR-93-5p	Osterix	Increased in serum of osteoporosis patients,[16] and inhibits bone mineralization.[91]
hsa-miR-16-5p	RANKL	Inhibits osteoclastogenesis in vitro.[92]

Table 2. Baseline Characteristics of the Population Available for the Cross–Sectional Analysis Comparing Pre- and Postmenopausal Women

	Cross-sectional analysis	Premenopausal women	Postmenopausal women	*p* value
Number of subjects	682	99	583	–
Age, years (min–max)	65.5 (44–94)	48.7 (44–55)	68.2 (45–94)	*
Weight, kg (min–max)	62 (38–117)	62 (44–117)	62 (38–116)	NS
Height, cm (min–max)	159 (138–179)	162 (148–179)	158 (138–178)	*
BMI, kg/cm^2 (min–max)	24.7 (15.4–46.8)	23.6 (17.5–42.7)	24.9 (15.4–46.8)	*
Smoker, number of subjects	48	12	36	NS
Prevalent fracture, number of patients	123	1	122	*
Median time from prevalent fracture to baseline visit, years (min–max)	4.6 (0.1–12.9)	0.7 (–)	4.6 (0.1–12.9)	*
Glomerular filtration rate by CKD EPI, mL/min/1.73 m^2 (min–max)	84 (26–115)	95 (66–115)	82 (26–114)	NS
Age at menopause, years (min–max)	50.5 (29–59)	–	50.5 (29–59)	–
Femoral neck BMD, g/cm² (min–max)	0.710 (0.438–1.157)	0.800 (0.575–1.157)	0.694 (0.438–1.098)	*
Total hip area BMD, g/cm² (min–max)	0.853 (0.414–1.263)	0.955 (0.692–1.263)	0.836 (0.414–1.192)	*
L1–L4 BMD, g/cm² (min–max)	0.912 (0.585–1.495)	1.004 (0.758–1.396)	0.896 (0.585–1.495)	*
Radius Tt.vBMD, mg/cm^3 (min–max)	282.2 (101.9– 515.8)	341.4 (229.8 – 489.0)	272.2 (101.9 – 515.8)	*
Radius Ct.vBMD, mg/cm^3 (min–max)	813.6 (557.0–1008.7)	909.0 (798.0–1008.7)	797.4 (556.9– 1004.8)	*
Radius Tb.vBMD, mg/cm^3 (min–max)	142.1 (17.5–274.5)	163.9 (86.2–274.5)	138.4 (17.5–264.0)	*
Tibia Tt.vBMD, mg/cm^3 (min–max)	256.0 (109.0–419.3)	301.6 (209.0–419.3)	248.4 (109.0–417.4)	*
Tibia Ct.vBMD, mg/cm^3 (min–max)	760.0 (376.7– 986.5)	895.4 (716.6–986.5)	737.3 (376.7–976.6)	*
Tibia Tb.vBMD, mg/cm^3 (min–max)	148.9 (31.9–254.7)	164.2 (96.0–251.5)	146.4 (31.9–254.7)	*
CTX, μg/L (min–max)	0.466 (0.057–1.470)	0.374 (0.107–1.030)	0.481 (0.057–1.470)	*
Osteocalcin, ng/mL (min–max)	26.8 (4.2–90.2)	20.5 (7.9–56.2)	27.9 (4.2–90.2)	*
P1NP, ng/mL (min–max)	49.6 (8.1–139.7)	43.8 (18.9–123.6)	50.5 (8.1–139.7)	*
BAP, UI/L (min–max)	37.8 (10.1–88.6)	30.2 (12.1–59.8)	39.0 (10.1–88.6)	*

*p value considered as significant if <0.05.

Tt.vBMD = total volumetric bone mineral density; Ct.vBMD = cortical volumetric bone mineral density; Tb.vBMD = trabecular volumetric bone mineral density; BAP = bone alkaline phosphatase.

L1 to L4 BMD), like the vBMD (20% for total radius and 18% for total tibia). Similarly, BTMs were higher in the postmenopausal women (22% higher for CTX, 27% for osteocalcin, 13% for P1NP, and 23% for BAP).

Sample hemolysis

With regard to the blood samples, macroscopic analysis showed that a few samples were affected by hemolysis: We observed that 64 (9%) samples had low levels of hemolysis and 4 (0.6%) samples had moderate levels of hemolysis). Removing the 68 hemolytic samples from the analysis did not change the results.

Serum levels of miRNAs and bone variables in the cross-sectional analysis

Table 3 shows the relative serum levels of each miRNA as a function of menopausal status. None of the miRNAs showed a significant difference by the menopausal status after adjustment for age. Besides, we also compared other subgroups according to the duration of menopause ($\leq$5 years and $>$5 years), to the BMD category (osteoporotic women with *T*-score $<$-2.5 DS versus $>$-2.5 DS, and osteopenic women with *T*-score $<$-2.5 DS versus $>$-1 DS), or to the occurrence of fracture or not. None of these subgroup analyses revealed any significant difference after adjustment for age. A subgroup analysis, assessing each Genant grade separately for prevalent vertebral fracture, showed no significant differences in the serum levels of the miRNAs after adjustment for age (results not shown).

Moreover, nine miRNAs did have significantly different values considering the presence or absence of an antiosteoporotic treatment at baseline. All these differences disappeared after adjustment for age (results in Supplementary Table S3).

Clinical characteristics

We observed a significant association between prevalent fractures and the serum level of 22 miRNAs that disappeared after age adjustment (Table 4). Further stratifying the dataset by menopausal status did not improve correlation to prevalent fracture in age-adjusted data.

This may be confounded by the observation that age appeared to be a parameter that was consistently positively correlated with serum miRNA levels across all 32 miRNAs analyzed (correlation coefficient from 0.09 to 0.49). This was also the case for miR-16-5p, which had the highest correlation coefficient between age and serum levels ($r = -0.49$, $p < 0.0001$; Fig. 2). We found height, weight, and BMI to be weakly correlated with miRNA serum levels ($r = 0.10$ to 0.15).

Bone turnover markers

Of the bone remodeling markers evaluated, only osteocalcin was significantly, albeit weakly, associated with 21 of the 32

Table 3. Comparison of Baseline Relative Serum Levels of the miRNAs (Medians) Regarding the Menopausal Status in the Cross-Sectional Analysis (*p* Value and Age-Adjusted *p* Value if Significant in Univariate Analysis)

	Relative levels (medians)			
miRNAs	Premenopausal ($n = 99$)	Postmenopausal ($n = 583$)	*p* Value	*p* Value adjusted for age
hsa-miR-133a-3p	5.4	3.2	0.002	0.90
hsa-miR-20a-5p	1.5	1.0	0.0003	0.87
hsa-miR-25-3p	1.5	0.9	<0.0001	0.92
hsa-miR-100-5p	2.1	1.3	0.05	0.50
hsa-miR-133b	3.1	2.6	0.03	0.72
hsa-miR-214-3p	2.7	2.5	0.12	
hsa-miR-26a-5p	1.7	1.1	0.04	0.93
hsa-miR-103a-3p	1.2	1.2	0.81	
hsa-miR-145-5p	2.3	1.4	0.09	
hsa-miR-21-5p	1.6	1.3	0.50	
hsa-miR-29a-3p	1.6	1.4	0.29	
hsa-miR-106a-5p	1.5	1.1	0.10	
hsa-miR-146a-5p	1.2	1.3	0.84	
hsa-miR-221-5p	0.7	0.2	0.0002	0.80
hsa-miR-29b-3p	1.0	1.6	0.03	0.05
hsa-miR-122-5p	1.6	1.0	0.004	0.49
hsa-miR-148a-3p	1.2	1.6	0.63	
hsa-miR-222-3p	1.2	1.2	0.40	
hsa-miR-338-3p	2.3	2.4	0.97	
hsa-miR-124-3p	0.9	0.3	0.02	0.39
hsa-miR-155-5p	1.7	2.0	0.82	
hsa-miR-223-5p	5.4	3.3	0.02	0.80
hsa-miR-34a-5p	0.6	12.1	0.45	
hsa-miR-125b-5p	1.5	0.9	0.002	0.91
hsa-miR-17-5p	2.2	1.3	0.02	0.99
hsa-miR-23a-3p	1.5	1.0	0.0006	0.70
hsa-miR-503-5p	1.3	0.3	0.11	
hsa-miR-127-3p	8.0	7.5	0.53	
hsa-miR-204-5p	8.5	2.3	0.006	0.76
hsa-miR-24-3p	1.5	0.9	<0.0001	0.56
hsa-miR-93-5p	1.6	1.1	<0.0001	0.70
hsa-miR-16-5p	1.5	0.9	<0.0001	0.36

miRNAs (with coefficients of correlation from −0.09 to −0.19). The other bone remodeling biomarkers (CTX, P1NP, and BAP) were not associated with the serum levels of the miRNAs.

DXA and HRpQCT

In univariate analysis, 26 and 19 miRNAs out of the 32 miRNAs were associated with neck and total hip BMD, respectively, with correlation coefficients from +0.08 to +0.23 and from +0.08 to +0.20, but not significantly after adjustment for age. Only two miRNAs (miR-214-3p and miR-222-3p) were significantly associated with lumbar spine BMD, but not after adjustment for age. Supplementary Table S4 shows the absence of significant association between BMD and miRNAs by linear regression.

In univariate analysis, 27 and 29 miRNAs were associated with the radius and tibia vBMD, respectively, with correlation coefficients from +0.10 to +0.22 and from +0.10 to +0.26. These were not significant after age adjustment.

Serum level of miRNAs and incident fragility fractures in postmenopausal women in the prospective analysis

Subjects' characteristics

As of January 1, 2015, incident fracture data became available for 577 postmenopausal women out of the 583 initially analyzed. The mean duration of follow-up was 7.7 years (0 to 9.3). Of these 577 women, 108 had at least one incident fracture: 32 women with vertebral fracture(s) (4 grade 1 according to Genant score, 16 grade 2, and 12 grade 3), 14 hip fractures, 8 proximal humerus fractures, 24 wrist fractures, and 12 tibia fractures. The other sites were metatarsal bone ($n = 5$), ribs ($n = 5$), elbow ($n = 1$), navicular ($n = 1$), patella ($n = 4$), collarbone ($n = 1$), and pelvis ($n = 1$).

Table 5 provides a summary of the patients' characteristics in relation to incident fracture. Women with incident fracture were older (70.8 versus 67.6 years old) and had significantly lower BMD (9% lower for the neck, 6% for the total hip area, and 5% for L1 to L4). Similarly, they had lower vBMD (12% lower for total radius and 10% for total tibia). BTMs were not significantly different between groups.

Association with incident fractures

Only 2 of the 32 miRNAs (miR-145-5p and miR-503-5p) were associated with incident fracture. However, this association was not observed following age-adjustment (Table 6). A subgroup analysis, assessing each Genant grade separately for incident vertebral fracture, showed no significant differences in the serum levels of the miRNAs after adjustment for age (results not shown).

Table 4. Comparison of Baseline Relative Serum Levels of the miRNAs (Medians) Regarding the Occurrence of Prevalent Fracture in the Cross-Sectional Analysis (Odds Ratio and 95% Confidence Interval by Logistic Regression, *p* Value and Age-Adjusted *p* Value if Significant in Univariate Analysis)

	Relative levels (medians)				
miRNAs	Without fracture ($n = 559$)	With fracture(s) ($n = 123$)	Odds ratio [95% CI]	*p* Value	*p* Value adjusted for age
hsa-miR-133a-3p	3.6	2.4	0.99 [0.97–1.01]	0.07	
hsa-miR-20a-5p	1.1	0.7	0.79 [0.68–0.92]	<0.0001	0.65
hsa-miR-25-3p	1.0	0.7	0.86 [0.77–0.96]	<0.0001	0.50
hsa-miR-100-5p	1.4	1.1	0.93 [0.86–1.00]	0.01	0.77
hsa-miR-133b	2.8	2.4	0.99 [0.97–1.00]	0.10	
hsa-miR-214-3p	2.7	2.0	0.99 [0.99–1.00]	0.17	
hsa-miR-26a-5p	1.3	0.9	0.91 [0.82–0.99]	0.001	0.71
hsa-miR-103a-3p	1.2	0.9	0.90 [0.83–0.99]	0.002	0.99
hsa-miR-145-5p	1.7	1.0	0.96 [0.92–0.99]	0.003	0.75
hsa-miR-21-5p	1.3	1.0	0.92 [0.85–0.99]	0.005	0.18
hsa-miR-29a-3p	1.4	1.1	0.95 [0.89–0.99]	0.01	0.18
hsa-miR-106a-5p	1.2	0.8	0.85 [0.75–0.96]	<0.0001	0.98
hsa-miR-146a-5p	1.4	1.0	0.91 [0.85–0.99]	0.001	0.94
hsa-miR-221-5p	0.2	0.1	0.99 [0.98–0.99]	0.04	0.73
hsa-miR-29b-3p	1.7	1.4	0.98 [0.94–1.02]	0.33	
hsa-miR-122-5p	1.1	0.6	0.90 [0.82–0.99]	<0.0001	0.70
hsa-miR-148a-3p	1.7	1.0	0.99 [0.98–0.99]	0.03	0.75
hsa-miR-222-3p	1.3	0.9	0.92 [0.84–0.99]	0.001	0.24
hsa-miR-338-3p	2.5	2.1	1.00 [0.99–1.00]	0.11	
hsa-miR-124-3p	0.4	0.3	1.00 [0.99–1.00]	0.37	
hsa-miR-155-5p	2.0	1.6	0.95 [0.91–0.99]	0.04	0.52
hsa-miR-223-5p	3.7	2.3	0.99 [0.98–0.99]	0.04	0.91
hsa-miR-34a-5p	7.6	13.1	0.99 [0.99–1.00]	0.57	
hsa-miR-125b-5p	1.1	0.7	0.83 [0.73–0.95]	0.0001	0.73
hsa-miR-17-5p	1.5	1.0	0.93 [0.88–0.98]	0.02	0.35
hsa-miR-23a-3p	1.1	0.7	0.85 [076–0.95]	<0.0001	0.87
hsa-miR-503-5p	0.4	1.9	0.99 [0.99–1.00]	0.76	
hsa-miR-127-3p	8 .1	5.8	1.00 [0.99–1.00]	0.16	0.89
hsa-miR-204-5p	3.5	3.8	1.00 [0.99–1.00]	0.96	
hsa-miR-24-3p	1.1	0.6	0.86 [0.76–0.96]	<0.0001	0.69
hsa-miR-93-5p	1.2	0.8	0.86 [0.77–0.97]	<0.0001	0.44
hsa-miR-16-5p	1.0	0.7	0.99 [0.98–0.99]	<0.0001	0.53

Discussion

Although miRNAs have been commonly reported to be promising biomarkers because of their differential expression in many disease states, there are a limited number of studies that follow-up these findings in large cohorts. We assessed 32 miRNAs with previously reported bone function and/or association with osteoporotic fractures in a large cohort of 682 women from the OFELY Cohort. After systematically assessing each clinical parameter and markers, we identified no significant association between prevalent or incident fractures, BTM, DXA, and HRpQCT parameters for the 32 miRNAs investigated.

We believe that the measurement of microstructure in our study represents an asset. Even if we found no association between those 32 microRNAs and fracture or vBMD, an association with microstructure could have arisen and raise mechanistic hypotheses, but also would have threatened the validity of some results. This was not the case; all our results were consistent, so we can consider these results robust.

The assessment of bone metabolism and bone fragility is still challenging. Indeed, histomorphometry, which is considered the reference method, cannot be used in daily practice. In this context, the development of circulating biomarkers is attractive. However, one must keep in mind that these miRNAs represent a snapshot of all available cells in the circulation, not only blood cells but also cells from tissue other than bone, which complicates their scope and interpretation.

Rather than an agnostic screening approach using microarrays, these miRNAs were selected based on positive associations observed in previously published studies pertaining to bone density or the occurrence of fractures in small sample sizes (see Table 1 for more details).[16,33] Thus, no validation study has been conducted as this work relies on previous promising data published on the 32 selected miRNAs. For example, miR-21-5p was previously demonstrated by Seeliger and colleagues to be increased in the serum of osteoporotic women in comparison with nonosteoporotic women. Panach et al. also showed increased serum levels in osteoporotic women with fracture in comparison with controls without fracture.[16,33]

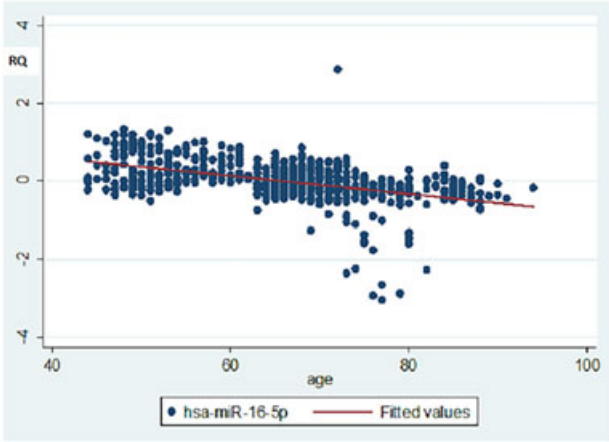

Fig. 2. Regression analysis of serum level of miR-16-5p and age.

A notable target of miR-21-5p is *PDCD4*, which is known to inhibit c-Fos, leads to enhanced osteoclastogenesis. In agreement with these authors, we found a significant association of the miR-21-5p level in serum with bone variables and prevalent factures that did not remain significant after age-adjustment. MiR-125b-5p was shown to be increased in the serum of osteoporotic and/or women with fractures in comparison with control.[16,33,34] These observations may be explained by the conclusions of Mizuno and colleagues, whereby miR-125b-5p targeting ERB-β2 leads to the inhibition of osteoblastogenesis through reduced cell proliferation.[35] In the present study, the circulating level of miR-125b-5p was decreased in postmenopausal women and significantly associated with densitometric parameters and the occurrence of prevalent fractures. Thus, despite the significant association between circulating level of several miRNAs and bone variables, age appears to be a confounding factor for the association of these miRNAs with osteoporotic fracture.

One of the strengths in our study is that there are a number of measures that have been carefully instated to minimize potential sources of variation in the study. For instance, all experiments, including RNA extraction and cDNA synthesis, have been performed by the same person (EF); this is also true for bone turnover marker measurements. Further, the use of the OFELY Cohort allows the control of a large part of the preanalytic variation, such as conditions of sample collection: Blood samples were collected between 8:00 a.m. and 9:30 a.m. after an overnight fast. This fortuitously allows minimizing variations introduced by miRNAs that may be affected by the circadian rhythm.[36] In addition, all samples were collected by the same laboratory, promoting uniform treatment in terms of collection and storage. We also used appropriated statistics in line with the nonparametric distribution of the miRNA levels in serum. We have also systematically adjusted for age, which is known to greatly influence the variations in the level of circulating miRNAs.[37–41]

There may be several explanations for the discrepancies between the previously published studies and the present results. First, we systematically adjusted for age, which was not always performed in other studies.[16,33] This is not surprising, given that miRNAs are known to be temporally expressed across

Table 5. Baseline Characteristics of the Population Available for the Prospective Analysis Comparing Women With or Without Fracture

Initial characteristics	Incident fracture	No incident fracture	*p* Value
Number of subjects	108	469	
Age, years (min–max)	70.8 (49–94)	67.6 (46–91)	*
Weight, kg (min–max)	62 (39–116)	62 (38–106)	NS
Height, cm (min–max)	159 (142–176)	158 (138–178)	NS
BMI, kg/cm² (min–max)	24.5 (16.4–46.8)	24.9 (15.4–42.6)	NS
Smoker, number of subjects	10	26	NS
Prevalent fracture at baseline, number of patients	36	85	NS
Median time from baseline to incident fracture, years (min–max)	3.8 (0.1–8.3)	–	–
Glomerular filtration rate by CKD EPI, mL/min/1.73 m^2 (min–max)	83 (47–114)	82 (26–114)	NS
Age at menopause, years (min–max)	51 (29–58)	50 (36–59)	NS
Femoral neck BMD, g/cm² (min–max)	0.664 (0.488–0.906)	0.700 (0.438–1.098)	*
Total hip area BMD, g/cm² (min–max)	0.797 (0.521–1.087)	0.844 (0.414–1.192)	*
L1L4 BMD, g/cm² (min–max)	0.857 (0.586–1216)	0.904 (0.585–1.494)	*
Radius Tt.vBMD, mg/cm^3 (min–max)	245.6 (122.9–482.1)	278.1 (101.9–515.8)	*
Radius Ct.vBMD, mg/cm^3 (min–max)	771.9 (556.9–956.4)	803.0 (572.6–1004.8)	*
Radius Tb.vBMD, mg/cm^3 (min–max)	123.2 (50.3–264.0)	141.8 (17.5–262.8)	*
Tibia Tt.vBMD, mg/cm^3 (min–max)	228.3 (124.2–371.2)	252.8 (109.0–417.4)	*
Tibia Ct.vBMD, mg/cm^3 (min–max)	702.6 (437.7–912.7)	744.6 (376.7–976.6)	*
Tibia Tb.vBMD, mg/cm^3 (min–max)	133.6 (52.0–212.6)	149.3 (31.9–254.7)	*
CTX, μg/L (min–max)	0.463 (0.057–1.16)	0.484 (0.062–1.470)	NS
Osteocalcin, ng/mL (min–max)	28.6 (4.2–90.2)	27.7 (6.5–86.2)	NS
P1NP, ng/mL (min–max)	49.0 (8.1–138.6)	21.5 (10.0–139.7)	NS
BAP, UI/L (min–max)	39.3 (15.2–69.4)	38.8 (10.1–88.6)	NS

*p-value considered as significant if <0.05.

Tt.vBMD = total volumetric bone mineral density; Ct.vBMD = cortical volumetric bone mineral density; Tb.vBMD = trabecular volumetric bone mineral density; BAP = bone alkaline phosphatase.

Table 6. Comparison of Baseline Relative Serum Levels of the miRNAs (Medians) Regarding the Occurrence of Incident Fracture in the Prospective Analysis (Odds Ratio and Confidence Interval by Logistic Regression, *p* Value and Age-Adjusted *p* Value if Significant in Univariate Analysis)

	Relative levels (medians)				
miRNAs	Without fracture ($n = 469$)	With fracture(s) ($n = 108$)	Odds ratio [95% CI]	*p* Value	*p* Value adjusted for age
hsa-miR-133a-3p	3.4	2.5	0.99 [0.98–1.01]	0.08	
hsa-miR-20a-5p	1.0	0.9	0.90 [0.77–1.04]	0.23	
hsa-miR-25-3p	0.9	0.8	0.92 [0.81–1.04]	0.21	
hsa-miR-100-5p	1.4	1.2	0.93 [0.86–1.02]	0.22	
hsa-miR-133b	2.6	2.6	0.99 [0.99–1.00]	0.56	
hsa-miR-214-3p	2.2	2.2	0.99 [0.98–1.01]	0.48	
hsa-miR-26a-5p	1.2	1.0	0.93 [0.84–1.03]	0.06	
hsa-miR-103a-3p	1.2	1.1	0.95 [0.87–1.03]	0.42	
hsa-miR-145-5p	1.5	1.3	0.96 [0.91–1.00]	0.05	0.27
hsa-miR-21-5p	1.3	1.2	0.93 [0.83–1.03]	0.54	
hsa-miR-29a-3p	1.4	1.3	0.98 [0.91–1.05]	0.39	
hsa-miR-106a-5p	1.1	1.0	0.95 [0.85–1.06]	0.35	
hsa-miR-146a-5p	1.3	1.2	0.95 [0.88–1.03]	0.19	
hsa-miR-221-5p	0.2	0.1	0.99 [0.99–1.00]	0.34	
hsa-miR-29b-3p	1.7	1.5	0.97 [0.92–1.03]	0.73	
hsa-miR-122-5p	1.0	0.9	1.01 [0.95–1.07]	0.48	
hsa-miR-148a-3p	1.6	1.5	0.99 [0.97–1.02]	0.86	
hsa-miR-222-3p	1.3	1.1	0.94 [0.85–1.04]	0.25	
hsa-miR-338-3p	2.4	2.9	0.99 [0.97–1.01]	0.67	
hsa-miR-124-3p	0.3	0.3	0.99 [0.99–1.00]	0.39	
hsa-miR-155-5p	2.0	2.0	0.97 [0.93–1.01]	0.56	
hsa-miR-223-5p	3.6	2.3	0.99 [0.97–1.00]	0.25	
hsa-miR-34a-5p	13.0	10.4	0.99 [0.99–1.00]	0.92	
hsa-miR-125b-5p	1.0	0.8	0.91 [0.80–1.04]	0.10	
hsa-miR-17-5p	1.3	1.2	0.97 [0.92–1.01]	0.62	
hsa-miR-23a-3p	1.0	0.9	0.94 [0.84–1.05]	0.29	
hsa-miR-503-5p	0.5	0.1	0.98 [0.98–0.99]	0.03	0.51
hsa-miR-127-3p	8.1	6.8	0.99 [0.99–1.00]	0.23	
hsa-miR-204-5p	3.0	1.8	1.00 [0.99–1.00]	0.72	
hsa-miR-24-3p	1.0	0.9	0.94 [0.83–1.06]	0.31	
hsa-miR-93-5p	1.1	1.0	0.92 [0.80–1.05]	0.29	
hsa-miR-16-5p	0.9	0.8	0.86 [0.73–1.02]	0.06	

different time points in cell cycles as well as in development. In addition, the association between miRNA serum levels and aging has been reported in several studies to date in a wide range of physiological processes.[37–41] For example, it has been established that miR-188 is a key regulator in the age-related switch between osteogenesis and adipogenesis in bone marrow mesenchymal stem cells.[42]

Second, we may assume that the influence of the circulating miRNAs differs over the life course. It seems that the serum levels of some miRNAs may be influenced by the occurrence of recent fracture.[17] miRNAs may also have a stronger influence during growth or at a specific time around menopause. In addition, most published articles for miRNA as biomarkers are reported in the field of oncology. Tumors are known to have aberrant extravasation, and cancer patients have been reported to have high numbers of exosomes and cell-free miRNAs in their circulation systems.[43] Genomic instability in carcinogenesis may greatly modulate the level of circulating miRNAs, enabling a more accurate detection of differential expression. Subtle changes induced by bone disease may be less readily detected. However, we have shown here that significant variations in the level of several miRNAs were associated with bone alterations, but were not observed after age-adjustment. Therefore, it is possible that miRNA changes associated with aging impairs the accurate assessment of miRNA changes during the progression of osteoporosis.[44]

Since the selection of our miRNA panel, several more miRNAs have been reported to play a role in bone fracture and osteoporosis in humans. Yavropoulou and colleagues preselected 14 miRNAs based on a literature search.[20] They found a significantly higher serum level of miR-2861 in osteoporotic women ($n = 70$) in comparison with healthy controls ($n = 30$). They also identified miR-124-3p to be higher, and miR-21-5p, miR-23a-3p, and miR-29a-3p to be lower in osteoporotic women (these four miRNAs were included in our own selection). Using a miRNA microarray, You et al. identified miR-27a to have a significantly reduced serum level in osteoporotic patients in comparison with healthy controls ($n = 5$ and $n = 5$, respectively, for the microarray analysis) and confirmed their findings in 81 postmenopausal osteoporotic women in comparison with 74 healthy premenopausal women.[45] Garmilla-Ezquerra and colleagues[46] identified 13 miRNAs differentially expressed in

bone samples (8 women with hip fragility fractures and 8 with hip osteoarthritis). They confirmed their findings for two miRNAs in 38 bone samples (19 women with fractures and 19 women without): miR-187 was higher in the nonfracture group, whereas miR-518f was higher in bone samples from the fracture group. Of note, in these studies with a small sample size, the most relevant outcome—fracture—was not addressed. Most recently, miR-144-3p has been reported to regulate osteoclastogenesis through targeting RANK, and is downregulated in serum from osteoporosis patients.[34] Although this study also reported increased levels of miR-24-3p, miR-27a-3p, miR-100, miR-125b, miR-145-5p, and miR-122a in the serum of osteoporosis patients, the study involved only 60 subjects (15 nonosteoporosis and 45 osteoporosis patients). In the absence of significant association, the concern of inadequate sample size might be raised. Our study, however, is much larger than all these studies with positive results. We believe that the critical evaluation of these miRNAs is an important preclinical step. So far, to our knowledge, the largest study to date on serum miRNA levels in osteoporosis was assessed in a cohort of 230 subjects overall,[47] whereas the average size in the other studies was roughly from 20 to 100.

In addition to the aforementioned challenges, as yet there is no clear consensus on the most appropriate reference for normalization to adjust for serum miRNAs as detected by qRT-PCR in relative quantification. For example, miR-16 is commonly used as an endogenous control in serum studies; however, it is known to be affected by hemolysis.[48] Further, miR-16-5p is also well-described in cancer cell colonization in bone marrow, possibly through the regulation of TGFβ and hedgehog signaling.[49,50] This highlights one of the many challenges in serum miRNAs analysis, in which endogenous controls are disease-specific. We attempted to identify a candidate endogenous control using GeNorm, which is a commonly cited tool for selecting reference genes for qRT-PCR.[51] This method identified miR-93-5p. However, correlation strengths were not improved (results not shown); the possible involvement of this miRNA in bone is also questionable. The use of an external spike-in control is another commonly employed method of normalization for serum miRNAs, which removes this variable and provides a stable level of the control in all samples.

We also acknowledge that some parameters known to influence the levels of circulating miRNAs were not controlled or recorded: for example, tobacco use just before blood collection,[52] diet,[53] amount of physical activity and especially just before blood collection,[54,55] the use of some medications such as aspirin,[56] and lipoproteins levels.[57] Recently, a significant relationship between the baseline level of a few miRNAs and fracture has been observed in a group of patients with diabetes.[18] However, we were unable to examine this in our cohort because the prevalence of diabetes was less than 5%. Finally, the OFELY Cohort is largely ethnocentric, with the majority of subjects of French Caucasian background. This, and taking into account dietary differences in France, may also account for some variations in the data.

In conclusion, we did not find evidence of an association between the serum levels of the 32 preselected miRNAs and BMD, BTMs, and fractures in the OFELY Cohort. Although the role of these miRNAs may be important in the cellular context, the detectable levels in serum do seem to be useful for determining bone mass and bone turnover in postmenopausal women. This study constitutes a key step in translating basic research into clinical practice, and in assessing the utility of miRNA biomarkers for osteoporosis and fracture risk.

Disclosures

All authors state that they have no conflict of interest..

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