Circulating microRNA Signatures in Patients With Idiopathic and Postmenopausal Osteoporosis and Fragility Fractures

Roland Kocijan, Christian Muschitz, Elisabeth Geiger, Susanna Skalicky, Andreas Baierl, Rainer Dormann, Fabian Plachel, Xaver Feichtinger, Patrick Heimel, Astrid Fahrleitner-Pammer, Johannes Grillari, Heinz Redl, Heinrich Resch, and Matthias Hackl*

Context: Established bone turnover markers do not reflect fracture risk in idiopathic male and premenopausal osteoporosis and the role of microRNAs (miRNAs) in these patients is currently unclear. miRNAs are a class of small non-coding RNAs that regulate gene expression and bone tissue homeostasis. They are considered a new class of endocrine regulators with promising potential as biomarkers.

Objective: Evaluation of circulating miRNA signatures in male and female subjects with idiopathic and postmenopausal osteoporotic low-traumatic fractures.

Design, Setting, and Patients: This was a case-control study of cross-sectional design of 36 patients with prevalent low-traumatic fractures and 39 control subjects

Main Outcome Measures: One hundred eighty-seven miRNAs were quantified in serum by qPCR, compared between groups and correlated with established bone turnover markers.

Results: Significant differences in serum levels of circulating miRNAs were identified in all three subgroups (46 in premenopausal, 52 in postmenopausal, 55 in male). A set of 19 miRNAs was consistently regulated in all three subgroups. Eight miRNAs [miR-152-3p, miR-30e-5p, miR-140-5p, miR-324-3p, miR-19b-3p, miR-335-5p, miR-19a-3p, miR-550a-3p] were excellent discriminators of patients with low-traumatic fractures, regardless of age and sex, with area under the curve values $>$ 0.9. The 11 remaining miRNAs showed area under the curve values between 0.81 and 0.89. Correlation analysis identified significant correlations between miR-29b-3p and P1NP, and miR-365-5p and iPTH, TRAP5b, P1NP and Osteocalcin, as well as $BMD_{L1–L4}$ and miR-19b-3p, miR-324-3p, miR-532-5p, and miR-93-5p.

Conclusions: Specific serum miRNA profiles are strongly related to bone pathologies. Therefore miRNAs might be directly linked to bone tissue homeostasis. In particular, miR-29b-3p has previously been reported as regulator of osteogenic differentiation and could serve as a novel marker of bone turnover in osteoporotic patients as a member of a miRNA signature. **(*J Clin Endocrinol Metab* 101: 4125–4134, 2016)**

Postmenopausal osteoporosis is the most common reason for low-traumatic fractures, but bone loss and low-traumatic fractures also occur in premenopausal women and in young males. In the absence of identifiable causes, bone loss in men is considered male idiopathic osteoporosis (MIO) (1). Although complex osteoblast dysfunctions and altered gene expressions were suggested in histomorphometric studies (2), serum bone turnover markers (BTM) were reported to be in normal range in MIO (3, 4). Low levels of sclerostin and down-regulate

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in USA

Received June 10, 2016. Accepted August 17, 2016.
First Published Online August 23, 2016

* Author Affiliations are shown at the bottom of the next page.

Abbreviations: aBMD, areal bone mineral density; AUC, area under the curve; BMD, bone mineral density; BMI, body mass index; BTM, bone turnover marker; Cq-value, quantification value; CTX, cross-linked C-telopeptide; CV, coefficient of variation; DEXA, dual-energy X-ray absorptiometry; iPTH, intact PTH; MIO, male idiopathic osteoporosis; miRNA, microRNA; MSC, mesenchymal stem cell; P1NP, N-terminal type 1 procollagen propeptide; Pre-MP, premenopausal study arm; Post-MP, postmenopausal study arm; RANKL, Receptor activator of NF-κB ligand; ROC, receiving operator characteristic; TRAP5b, tartrate-resistant acid phosphatase.

Dickkopf-1 were observed in MIO, but they do not seem to reflect alterations in bone metabolism or high fracture risk adequately (3, 5).

In line with MIO, in most cases no secondary causes for premenopausal osteoporosis can be found either. Areal bone mineral density (BMD) (aBMD) is not necessarily related to fracture risk in idiopathic osteoporosis in men and women, respectively. Consequently, most low-traumatic fractures occur in patients with osteopenic or normal aBMD and there is a substantial overlap in aBMD values of patients with and without low-traumatic fractures (6).

As a result, dual-energy X-ray absorptiometry (DXA) and BTMs are limited in discriminating between premenopausal women and men with or without prevalent low-traumatic fractures. In these cases, reliable tools for fracture risk prediction are needed.

MicroRNAs (miRNAs) are small noncoding RNAs that span between 19 and 24 nucleotide bases. They gain biological activity through base pairing to target mRNA (mRNA) molecules, thereby guiding a protein complex (termed RISC) in the 3′-untranslated region. Binding of RISC to the mRNA sequence either results in mRNA degradation or inhibition of translation (7).

miRNAs play essential roles in the regulation of various biological processes including bone formation, -resorption, -remodelling, and differentiation of bone cells (8). Putative links between a deregulation of these negative regulators for gene expression and the onset and progression of chronic diseases have been described (9–11). Recent studies have demonstrated that miRNAs are also among the most abundant RNA species in cell-free blood (12, 13), due to targeted secretion of miRNAs via vesicular budding or endosomal release (14). Specific circulating miRNAs that are altered during cellular senescence (15), recent low-trauma fractures (16) and nonrecent low-trauma fractures have been identified to influence osteogenesis in mesenchymal stem cells (MSCs). It has further been suggested that combinations of miRNAs that reflect different tissues and physiologic conditions ("signatures") might serve as valuable biomarkers for the early diagnosis of multifactorial diseases, to predict disease progression, and accurately determine the risk of adverse outcomes (17).

We hypothesized that the multifactorial nature of bone fragility in idiopathic osteoporosis could be characterized by a specific pattern of circulating miRNAs in patient serum.

The primary objective of this study was to identify circulating miRNAs as novel discriminators between patients with idiopathic low-traumatic fractures and healthy subjects without fractures.

Secondary objectives were to evaluate differences in miRNA regulation between MIO, premenopausal and postmenopausal osteoporosis and their association to BTMs, aBMD, as well as concomitant bone specific treatment.

Materials and Methods

Study design

In this case-control, single center study, miRNA levels were assessed in patients with idiopathic osteoporosis and low-traumatic fractures and matched healthy controls. Patients and healthy subjects were recruited at the Medical Department II for Rheumatology and Bone Diseases, St. Vincent Hospital Vienna. The study was performed in cooperation with the Department of Internal Medicine, Division of Endocrinology and Diabetes of the Medical University of Graz, the Ludwig Boltzmann Institute for Experimental and Clinical Traumatology Vienna, the Department of Biotechnology of the University of Natural Resources and Life Sciences Vienna and the TAmiRNA GmbH.

Subjects

Patients were considered as having idiopathic osteoporosis if they 1) were premenopausal women or men aged < 50 years who 2) had sustained low-traumatic fractures but had normal medical history, 3) or had sustained low-traumatic fractures and had osteopenic or normal BMD values. Postmenopausal women were included in the study, if they had sustained 1) low-traumatic fractures, independent of their T-score and 2) had no reason for secondary osteoporosis. Patients were included in the study if they had sustained at least one peripheral or vertebral low-traumatic fracture without any ascertainable reason. Fractures were considered as low-traumatic if they occurred after minor trauma or without identifiable trauma (18). Lateral and antero-posterior digital x-rays of the thoracic and lumbar spine were performed to diagnose vertebral fractures. Peripheral fractures were self reported by questionnaire. To avoid the effects of fracture healing on miRNA profiles, time span from study relevant procedures to last fractures had to be at least 6 months.

Bone biopsies, blood analyses, and clinical investigations were performed in all patients to detect neoplastic, inflammatory, genetic, or metabolic disorders. Current and previous drugs including current and previous bone-related therapy (bisphosphonates, denosumab, teriparatide, strontium ranelate, raloxifene), previous fractures including trauma history and medical history were recorded. Exclusion criteria were secondary causes

St. Vincent Hospital—Medical Department II (R.K., C.M., R.D., F.P., X.F., H.Res.), The VINFORCE Study Group, Academic Teaching Hospital of Medical University of Vienna, 1090 Vienna, Austria; TAmiRNA, GmbH (E.G., S.S, M.H..), 1190 Vienna, Austria; Department of Statistics and Operations Research (A.B.), University of Vienna, 1090 Vienna, Austria; Ludwig Boltzmann Institute for Experimental and Clinical Traumatology (R.K., P.H., H.Red.), 1200 Vienna, Austria; Karl Donath Laboratory for Hard Tissue and Biomaterial Research, Department of Oral Surgery (P.H.), Medical University of Vienna, 1090 Vienna, Austria; Department of Internal Medicine, Division of Endocrinology and Diabetes (A.F.-P.), Medical University of Graz, 8010 Graz, Austria; Christian Doppler Laboratory on Biotechnology of Skin Aging, Department of Biotechnology (J.G.), University of Natural Resources and Life Sciences Vienna, 1180 Viena, Austria; Austrian Cluster for Tissue Regeneration (H.Red., J.G.), Department of Traumatology, Medical University of Vienna, 1090 Vienna, Austria; and Medical Faculty of Bone Diseases (H.Red.), Sigmund Freud University–Vienna, 1020 Vienna, Austria

for osteoporosis including inflammatory diseases, genetic disorders affecting bone such as osteogenesis imperfecta, Ehlers-Danlos-syndrome and fibrous dysplasia, diabetes mellitus type 1 and 2, chronic obstructive pulmonary disease, chronic kidney and liver dysfunction, systemic glucocorticoid use and glucocorticoid-induced osteoporosis, eating disorders, HIV-infections, and any malignancy including plasmacytosis and lymphoma. For further data analysis, patients were divided into premenopausal women, postmenopausal women and men with low-traumatic fractures for data analyses.

MiRNA analyses were also performed in an age- and sex-matched healthy control group without low-traumatic fractures. To directly compare the subgroups, controls were also divided into premenopausal women, postmenopausal women, and men. Healthy subjects were randomly selected from the resident population. Subjects were included when they were in the age range of the patients and fit the sex ratio, independent of height, weight, body mass index (BMI), or a special matching algorithm. Subjects were excluded from the control group if they suffered from inflammatory disorders, bone diseases including osteoporosis, or kidney or liver dysfunction. Previous therapy affecting bone metabolism including anticatabolic and osteoanabolic drugs and glucocorticoids as well as low-traumatic fractures were further exclusion criteria for the control population. Participants of this study did not receive any financial compensation.

All patients and controls signed written informed consent forms prior to any study-related procedures. The study was approved by the local ethics committee (approval number: 201501_EK04) and was conducted in accordance with the Declaration of Helsinki.

Laboratory analyses

Blood samples were drawn after overnight fasting between 0800 and 1000 hours. All samples were immediately centrifuged and stored at −80°C. Intact PTH (iPTH), 25-hydroxyvitamin D, and TSH were measured by Abbott Architect platform. Bone turnover markers including Osteocalcin, intact N-terminal type 1 procollagen propeptide (P1NP), cross-linked C-telopeptide (CTX) were analyzed by electrochemiluminescence immunoassays. Intra-assay coefficients of variation (CV) are 2.1–4.9% for CTX, 2.6–3.0% for P1NP, 1.1–3.7% for iPTH, and 5.5–7.1% for 25-hydroxy vitamin D. Receptor activator of NF-κB ligand (RANKL) and osteoprotegerin were analyzed by microplate reader (Dynex Technologies), bone-specific alkaline phosphatase by chemiluminescence (IDS-ISYS), free T by radio immunoassay, tartrate-resistant acid phosphatase (TRAP5b) using enzyme immunoassay.

microRNA analysis

In total, 187 miRNAs and five controls were analyzed in the entire cohort of 75 individuals over a period of 4 weeks. All miRNAs were selected based on the results from previous studies (16, 17). The analytical quality of this high-dimensional microRNA dataset was assessed using synthetic spike-in controls. Uniform quantification values (Cq-values) obtained for all spike-ins demonstrated successful and homogeneous RNA isolation, reverse transcription and qPCR for all samples (Supplemental Figure 1). Hemolysis is a potential confounder of circulating miRNAs. Therefore, the presence of hemolysis can be assessed using the "hemolysis-index," which is based on the ratio of miR-23a-3p and miR-451a-5p (19). No samples had to be excluded due to hemolysis.

RNA extraction from serum samples

The miRNeasy Mini Kit (QIAGEN) was used to perform RNA-Isolation. Frozen serum samples at −80°C were thawed on ice and centrifuged at 12 000× *g* for 5 minutes. After centrifugation, 200 μL serum were mixed by vortexing with 1000 μL Qiazol, to which a mix of three synthetic spike-in controls (UniSp 2,4,5) had been added. After incubation at room temperature for 10 minutes, 200 μL chloroform were added to the homogenized sample, vigorously vortexed and incubated for 3 minutes. After centrifugation at 12 000× *g* for 15 minutes at 4°C, exactly 650 μL of upper aqueous phase were taken and glycogen (Ambion) was added to a final concentration of 50 μg/mL. Samples were then transferred to columns and further processed using the QIACube liquid handling robot. RNA was precipated with 750 μL Ethanol, triple washed with RPE-buffer, followed by RNA-elution in 30 μL nuclease-free water, and storage at −80°C.

microRNA qPCR analysis

First, 4 μL of isolated RNA were reversed transcribed using the Universal cDNA Synthesis Kit II (Exiqon). Synthetic cel-miR-39-3p was spiked at this step to control for enzyme inhibition. The reaction was incubated at 42°C for 60 minutes, and then heat inactivated at 95°C for 5 minutes. cDNA samples were stored at −20°C. Real Time quantitative PCR (RT-qPCR) analysis of 187 circulating miRNAs (Supplemental Data) was conducted using custom 384 well panels (Exiqon). For RT-qPCR analysis, cDNA samples were diluted 50-fold and 5μL were used in individual 10 μL PCR reactions using ExiLENT SYBR Green master mix and LNA-enhanced miRNA primer assays (Exiqon). PCR conditions were 95°C for 10 minutes, 45 cycles of denaturation (95°C, 10 sec) and annealing/elongation (60°C, 60 sec), and melting curve analysis on LC 480 Real Time PCR system (Roche). The second derivative method was used to calculate the cycle of Cq-values.

qPCR data analysis

Data quality for each sample was monitored using synthetic spike-in controls during RNA-Isolation (UniSp 2,4,5), cDNA synthesis (cel-miR-39-3p) and PCR amplification (UniSp3) to detect the presence of enzyme inhibitors and ensure equal purification efficiencies for all samples (Supplemental Figure 1). Background levels for each miRNA were generated on the basis of a nuclease-free water sample. Only miRNAs that elicited signals of more than five Cq-values lower than the background value were included in the analysis. Missing values were imputed by setting Cq-values to 42. The Normfinder algorithm was used to identify suitable references for data normalization (20). The mean Cq-value ("global mean") exhibited the highest stability (0.184) and was therefore used for normalization using the equation:

$$\text{normalized Cq(delta Cq or dCq)} = \text{mean Cq(all miRNAs)} - \text{miRNA Cq.}$$

Delta Cq suggests a relative $\log_2$-transformed measure for expression levels. Hemolysis was controlled by the ratio of miR-23a-3p and miR-451a-5p (19). Lipolysis as a potential confounder of circulating miRNA analysis was excluded on the basis

of RNA spike-in controls, given that successful RNA extraction could be confirmed for all analyzed samples (Supplemental Figure 1A).

aBMD by DXA and dual X-ray absorptiometry and laser technique

aBMD was assessed by dual energy x-ray absorptiometry (iDXA, GE Healthcare Lunar, software version Encore 13) at the total hip and the lumbar spine (L1–L4). Fractured vertebrae were excluded from analysis. Measurements were performed by one IOF-ISCD-certified physician (International Osteoporosis Foundation–International Society for Clinical Densitometry). Daily quality controls and cross-calibrations were conducted using standardized control phantoms (iDXA: aluminum spine phantom serial number 32619-2). The in vivo CV was reported to be 0.41% for lumbar spine and 0.53% for total hip (21). aBMD was expressed as T-score and g/cm^2.

Statistical analysis

Exploratory data analysis was performed using ClustVis, a web tool allowing principal component and heatmap analysis of multivariate dataset using different R packages (22). For all exploratory analyses normalized delta Cq values were used. First, miRNAs were ranked according to the CV of which the top 100 most variant miRNAs were selected. Principle component analysis was performed based on expression information from the top 100 miRNAs using single value decomposition. Clustering of top 100 miRNAs for heatmap visualization was performed using the Pearson correlation.

Differential expression analysis

Biostatistical analysis was performed using R (23), GraphPad Prism 5.0 and GenEx 6.0. The nonparametric two-tailed Mann-Whitney *U* test was used to test for differences between cases and controls in the three subgroups. The *P*-values obtained from the test were adjusted for multiple testing using Benjamini Hochbergs method for false-discovery rate calculation. An false discovery rate threshold of 5% (adj. $P < .05$) was used to call miRNAs differentially expressed.

Classification analysis

Receiving operator characteristic (ROC) curves and corresponding area under the curve (AUC) estimates were derived for individual miRNAs. Ninety-five percent confidence intervals for AUC-values are based on bootstrap samples with 2000 replicates. Bootstrapped true and false-positive rates were evaluated at all relevant thresholds to draw confidence bands for ROC curves. Logistic regression models were estimated for all possible models of size 5 from 9 previously found miRNAs. The performance of the models was evaluated by AUC values. To demonstrate the predictive power of previously discovered miRNAs, the AUC distribution was compared with distributions from models with randomly permutated case-control status.

Correlation analysis

Correlations between bone-specific circulating miRNAs, numerical clinical parameters (age and BMI), and BTMs was analyzed using Spearman rank correlation. Significance was tested against the null hypothesis of $r = 0$.

Table 1. Clinical Characteristics of the Study Cohort

Characteristic	OPO	CTRL	*P*-Value
No. of patients	36	39	
preMP/postMP/male	10/10/16	12/11/16	
Age, y	46.6 ± 13.0	46.6 ± 9.4	.986
Height, cm	170.3 ± 8.1	172.0 ± 10.3	.428
Weight, kg	71.3 ± 16.6	77.6 ± 13.6	.079
Body mass index, kg/m^2	24.6 ± 5.4	26.3 ± 4.4	.133
Vertebral fractures, mean	1.9 ± 2.2	0	
Peripheral fractures, mean	2.4 ± 4.4	0	
Smoking, %	36	23	
Alcohol, %	6	0	
Family history for fractures, %	22	0	
Previous Treatment, %	31	0	
Lactose intolerance, %	14	0	

OPO, Osteoporosis; CTRL, Control.

Results

Clinical characteristics

A total of 36 adult female and male patients with idiopathic osteoporosis (mean age, 46.6 ± 13.0 y) were included in the study: 10 premenopausal women (mean age, 39.0. ± 8.6), 10 postmenopausal women (mean age, 59.0 ± 11.4 y) and 16 men (mean age, 43.7 ± 11.1 y) with low-traumatic fractures. Vertebral fractures and peripheral low-traumatic fractures were observed in 61% and 50% of the patients, respectively. Twenty-two percent of patients sustained both vertebral and peripheral fractures. In total, 152 fractures were recorded (mean number of fractures, 4.2). Thirty-one percent of patients had received either anticatabolic (bisphosphonates, denosumab, n = 13) or osteoanabolic (teriparatide, n = 2) drugs within the last year. miRNAs were compared with 39 subjects without low-traumatic fractures (mean age, 46.6 ± 9.4 y): 12 premenopausal women (mean age, 42.5 ± 6.8 y), 11 postmenopausal women (mean age, 54.3 ± 7.4 y), and 16 men (mean age, 44.4 ± 9.6 y).

Patients with osteoporosis and healthy controls had similar age, height, weight, and therefore, BMI. The clinical characteristics of the cohort are listed in Table 1.

Evaluated markers of bone resorption and bone formation were in normal range for premenopausal women, postmenopausal women, and men with MIO, respectively. aBMD was decreased in all subgroups with osteopenic mean values. Biological characteristics for all cases are presented in Table 2.

Principal component analysis and hierarchical clustering

Based on the normalized dataset, miRNAs were ranked according to the observed relative variation across all 75 samples. The top 100 miRNAs, which showed the highest

Table 2. Serum and aBMD Values of the Osteoporotic Cohort

	Pre-MP	Post-MP	MIO	Pre-MP Versus Post-MP	Pre-MP Versus MIO	Post-MP Versus MIO
Treatment naive/BP/TPTD, n	5/5/0	3/6/1	13/2/1			
BTMs						
iPTH, pg/mL	34.9 ± 10.3	34.4 ± 13.5	26.3 ± 10.6	0.930	0.054	0.124
Serum CTX, ng/mL	0.191 ± 0.117	0.323 ± 0.153	0.340 ± 0.172	*0.045*	*0.015*	0.789
TRAP5b, U/L	1.9 ± 1.0	3.0 ± 0.8	2.8 ± 0.4	*0.023*	*0.036*	0.462
P1NP, μg/mL	29.4 ± 22.2	42.0 ± 26.5	47.2 ± 22.3	0.264	0.061	0.610
BALp, μg/L	16.1 ± 7.7	25.8 ± 12.7	22.3 ± 10.1	0.056	0.087	0.476
Osteocalcin, ng/mL	13.7 ± 7.4	20.4 ± 11.3	19.4 ± 8.9	0.136	0.089	0.823
25(OH) vitamin D, ng/mL	32.7 ± 11.5	42.8 ± 26.3	30.2 ± 15.0	0.288	0.625	0.189
Osteoprotegrin, pmol/L	2.3 ± 1.2	3.1 ± 1.9	2.5 ± 1.4	0.257	0.749	0.354
RANKL	0.071 ± 0.083	0.058 ± 0.084	0.078 ± 0.102	0.737	0.330	0.321
Free T, nmol/L	1.3 ± 0.9	1.0 ± 0.7	12.0 ± 4.9	0.392	*0.001*	*0.001*
aBMD Values						
L1–L4, g/cm^2	0.817 ± 0.313	0.719 ± 0.295	0.859 ± 0.377	0.479	0.762	0.302
L1–L4, T-score	−2.3 ± 0.9	−2.4 ± 1.8	−2.1 ± 1.5	0.818	0.720	0.641
Total hip, g/cm^2	0.646 ± 0.364	0.699 ± 0.272	0.764 ± 0.327	0.717	0.415	0.590
Total hip, T-score	−1.6 ± 1.2	−1.6 ± 1.2	−1.5 ± 1.1	0.914	0.876	0.777

Abbreviations: BP, previous bisphosphonate therapy; TPTD, previous teriparatide therapy.

Italic type indicates statistically significant findings.

variation, were selected to assess the effect of sex, fracture, and menopause on patterns of circulating miRNAs. Principal component analysis showed that prevalent fragility fractures were a strong effector of circulating miRNA levels compared with sex (Supplemental Figure 2, A and B). This was also confirmed by hierarchical cluster analysis (Supplemental Figure 2C), which showed that fracture status was the predominant effector of serum miRNA levels in this cohort compared with sex and menopause.

Differential expression analysis in subgroups

In fractured premenopausal women 46/187 miRNAs (14 up/32 down), in fractured postmenopausal women 53/187 miRNAs (20 up/33 down) and in male idiopathic patients 55/187 miRNAs were significantly regulated (23 up/32 down; see Figure 1A). An overlap of 11.0% (10 miRNAs) between premenopausal and postmenopausal women as well as male and postmenopausal women, and an overlap of 5.5% (5 miRNAs) between premenopausal women and males was found (see Figure 1B). A common set of 19 miRNA (20.9%), which were significantly regulated (adj-$P < .05$) in all three subgroups, was identified. Pretreatment did not significantly affect serum miRNA levels in this cohort (Supplemental Figure 3, A and B). We observed correlations between miRNA levels in pretreated and untreated subjects (Supplemental Figure 3C). MicroRNA levels in untreated and pretreated fracture patients are shown in Supplemental Figure 3D.

Nineteen circulating miRNAs with common regulation in premenopausal, postmenopausal, and male idiopathic osteoporosis

Table 3 lists IDs of 19 miRNAs, which were significantly regulated between patients with low-traumatic fractures and controls in all three subgroups. Three of 19 miRNAs (15%) were commonly up-regulated (miR-152-5p, miR-335-5p, miR-320a), whereas 16 (85%) were down-regulated. miRNAs have been found to be statistically significant discriminators between patients with low-traumatic fractures and controls. Eight miRNAs (miR-140-5p, miR-152-3p, miR-30e-5p, miR-324-3p, miR-335-3p, miR-19a-3p, miR-19b-3p, miR-550a-3p) had AUC values greater than 0.9 for the classification of fracture patients (Table 3 and Figure 2).

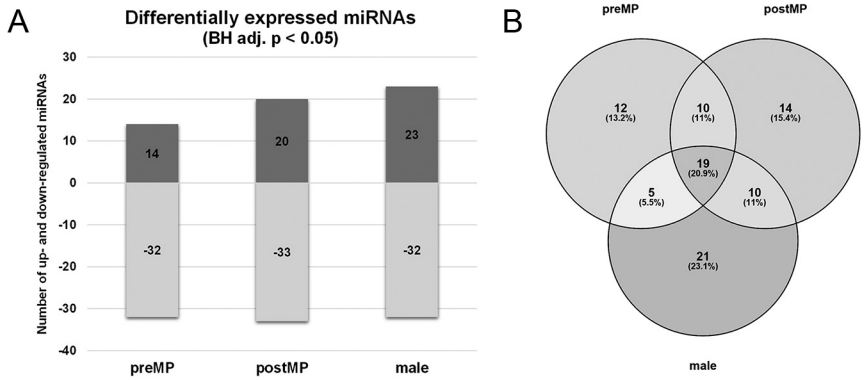

Figure 1. Differential expression analysis. Differential expression analysis was performed for each subgroup independently based on nonparametric *t* tests with adjustment for multiple testing using the Bonferroni-Holm method. The number of up- and down-regulated miRNAs are depicted as bar chart in panel A. Overlaps between subgroups is depicted in panel B as Venn diagram.

Table 3. Differential Expression Characteristics and Classification Performance of 19 microRNAs Between Patients With Fractures and Controls

miRNA ID	Fold Change (Group Mean OPO Versus Ctrl)	*P*-Value	Adjusted *P*-Value (Bonferroni-Holm)	AUC (95% CI)
hsa-miR-152-3p	1.79	1.95E-15	3.65E-13	0.962 (0.918–0.993)
hsa-miR-30e-5p	0.50	3.79E-15	7.05E-13	0.959 (0.901–0.997)
hsa-miR-140-5p	0.42	4.35E-14	8.01E-12	0.947 (0.900–0.983)
hsa-miR-324-3p	0.61	2.43E-14	4.50E-12	0.950 (0.885–0.994)
hsa-miR-19b-3p	0.43	8.81E-14	1.61E-11	0.944 (0.879–0.997)
hsa-miR-335-5p	2.16	2.27E-13	4.14E-11	0.939 (0.872–0.986)
hsa-miR-19a-3p	0.49	1.19E-12	2.15E-10	0.929 (0.856–0.983)
hsa-miR-550a-3p	0.45	2.89E-11	5.19E-09	0.909 (0.837–0.970)
hsa-miR-186-5p	0.59	1.22E-10	2.18E-08	0.898 (0.821–0.964)
hsa-miR-532-5p	0.53	1.34E-10	2.39E-08	0.898 (0.810–0.968)
hsa-miR-93-5p	0.58	1.40E-09	2.47E-07	0.879 (0.797–0.947)
hsa-miR-378a-5p	0.44	3.36E-09	5.88E-07	0.872 (0.785–0.940)
hsa-miR-320a	1.80	4.34E-09	7.55E-07	0.870 (0.781–0.942)
hsa-miR-16-5p	0.48	1.75E-08	3.01E-06	0.857 (0.761–0.940)
hsa-miR-215-5p	0.58	2.80E-08	4.76E-06	0.853 (0.761–0.930)
hsa-let-7b-5p	0.64	3.02E-08	5.08E-06	0.852 (0.756–0.929)
hsa-miR-29b-3p	0.63	1.25E-07	2.06E-05	0.838 (0.737–0.930)
hsa-miR-7-5p	0.51	5.02E-07	7.89E-05	0.824 (0.720–0.917)
hsa-miR-365a-3p	0.48	1.84E-06	2.74E-04	0.809 (0.707–0.898)

Abbreviation: CI, confidence interval.

Associations between BTM, aBMD, demographic data and levels of circulating miRNAs

The most significant correlations were found between miR-140-5p and BMI ($r = -0.55$), miR-29b-3p and P1NP ($r = 0.37$), miR-320a and BMI ($r = 0.34$), miR-365a-3p and iPTH ($r = 0.50$), TRAP5b ($r = 0.36$), P1NP ($r = 0.48$), and osteocalcin ($r = 0.38$). miR-19b-3p ($r = 0.37$), miR-324-3p ($r = 0.46$), miR-532-5p ($r = 0.36$), and miR-93-5p ($r = 0.40$) correlated significantly with lumbar spine aBMD. No significant associations to aBMD at the hip were found (Figure 3). Correlations between single miRNAs as well as BTM, aBMD and demographic data are shown in Supplemental Figure 4. In a separate correlation analysis between miRNAs and BTMs performed in patients with and without pretreatment, we observed higher correlations of four miRNAs (miR-550a-3p, miR-7-5p, miR-378a-5p, and miR-532-5p) to P1NP in pretreated subjects (Supplemental Figure 3E). In contrast, only miR-365a-3p showed a significant positive correlations to CTX in pretreated group (Supplemental Figure 3F).

Multivariate analysis—miRNA biomarker signatures for osteoporosis

The following putative miRNAs were used: miR-550a-5p, miR-550a-3p, miR-188-3p, miR-382-3p, miR-155-5p, miR-942-5p, miR-330-3p, miR-203a, and miR-181c-5p. miR-550a-3p was found to be significantly regulated in all three subgroups (Supplemental Figure 5A). miR-942-5p was found regulated in male and premenopausal osteoporosis, miR-188-3p and miR-181c-5p were regulated in the postmenopausal cohort and miR-382-3p in the male cohort (Supplemental Figure 5A).

Based on multivariate models, combinations of five from these nine miRNAs were generated and the performance with three independent negative-control datasets with permuted sample labels was challenged. A mean AUC value for these models of > 0.9 as well as a significant difference in the distribution of AUC-values between the real model and the permutation datasets (Supplemental Figure 5B) were observed.

Due to the high predictive power of all nine miRNAs, high AUC (>0.9) were found for all miRNA-combinations. The model with highest predictive power included miR-942-5p, miR-155-5p, miR-330-3p, miR-203a and miR-181c-5p (AUC: 0.97).

Discussion

In the present study we report levels of 187 miRNAs in a cohort of premenopausal women, men with idiopathic osteoporosis and postmenopausal women with low-traumatic fractures as well as healthy controls. Exploratory data analysis showed that history of low-traumatic fractures resulted in a distinct circulating miRNA pattern in the study population. The effect exceeded the effect of sex. This is in line with observations described by Mooney et al (24) in a study of young healthy volunteers, who showed that there are no significant differences in the detection rates and levels of miRNAs in plasma, respectively. A

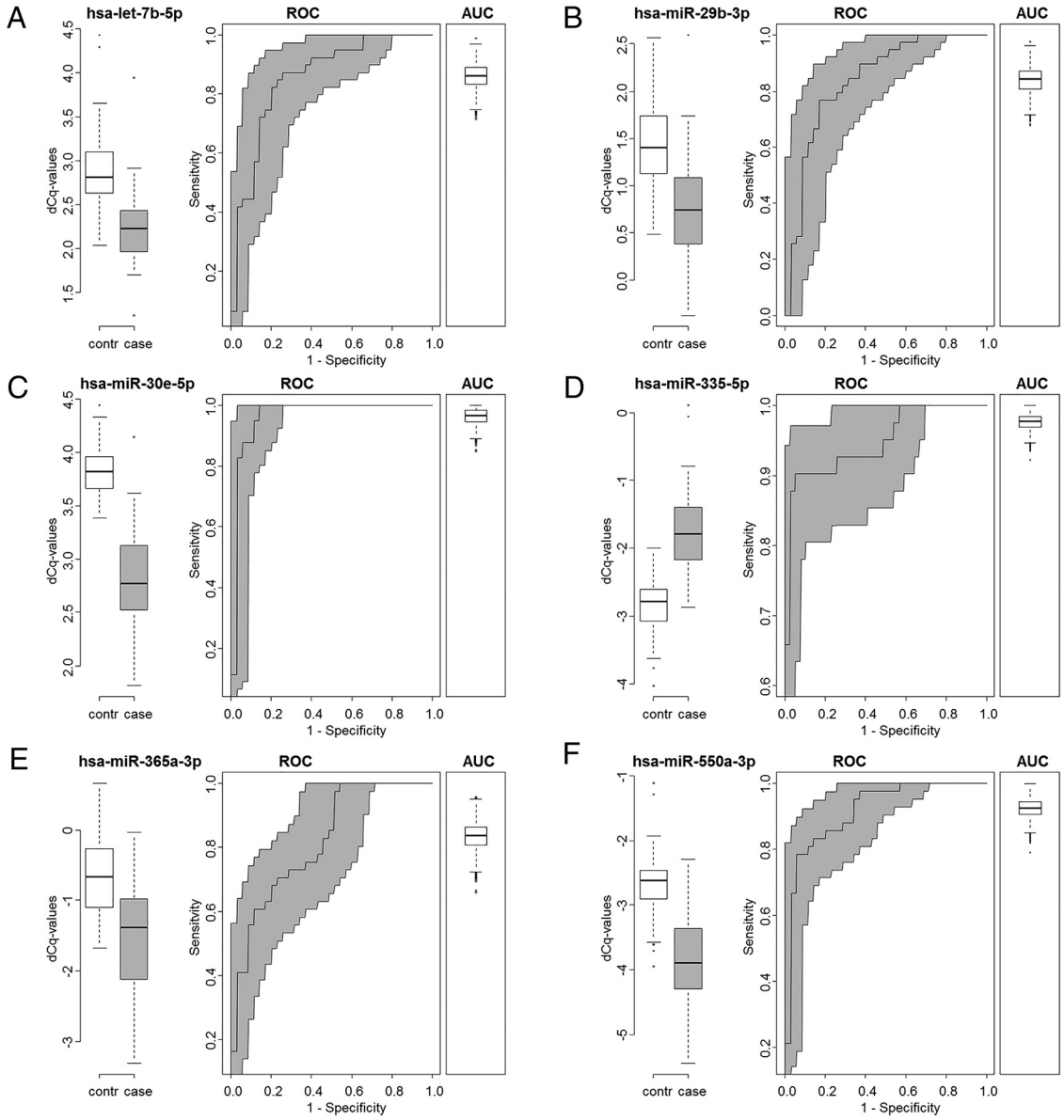

Figure 2. Effect size and classification performance observed for six selected microRNAs. A–F, Each plot depicts the distribution of normalized microRNA serum levels (δ-Cq value) in controls (n = 36) and cases with osteoporotic fractures (n = 39) as box-plot. Whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range from the box. The ability to differentiate between cases and controls was assessed using ROC analysis based on 2000 bootstrap samples. ROC-curves including 95% confidence interval (gray area) are shown. The distribution of the AUC values derived from bootstrap samples are shown on the right as box plot.

subset of 19 of 91 regulated miRNAs (21%) were found differently regulated in all patients with low-traumatic fractures when compared with subjects without fractures. The fact that the serum levels of miRNAs were altered regardless of sex and age, gives evidence of a typical miRNA-signature in patients with low-traumatic fractures. Highly significant AUC values of selected miRNAs revealed an almost perfect discrimination of patients with low-traumatic fractures from healthy controls. Multivariate classification models also resulted in significantly higher AUC values for the patient's dataset compared with a randomly selected permutation control, which supports the robustness and importance of our results.

Although a few studies have reported analyses of miRNAs in serum of patients with low BMD (25, 26) or recent low-traumatic fractures (16, 27, 28), these studies were either restricted to the analysis of very few preselected miRNAs or limited by sample size and therefore failed to provide a global view on miRNA changes during the pathology. Therefore, to confirm the diagnostic potential, we aimed to investigate miRNAs in a larger and heterogeneous osteoporotic study population.

miRNA	Age	BMI	Osteo-protegerin	BALp	iPTH	Serum CTX	TRAP5b	P1NP	Osteo-calcin	25 (OH) vitamin D	BMD L1-L4	BMD Hip
let-7b-5p	0.19	0.02	0.11	0.08	-0.11	0.07	0.10	0.14	0.05	0.06	0.12	-0.16
miR-140-5p	-0.14	-0.55*	0.20	-0.02	0.27	-0.14	-0.09	-0.26	-0.12	0.33	0.34	0.10
miR-152-3p	-0.10	0.24	0.21	-0.12	0.03	-0.14	-0.03	0.08	0.18	-0.04	-0.18	-0.04
miR-16-5p	0.17	0.24	0.08	-0.13	-0.29	0.00	-0.04	0.10	-0.02	0.06	0.15	-0.02
miR-186-5p	0.14	0.07	0.32	-0.15	0.09	-0.10	-0.28	-0.17	-0.20	0.10	0.17	0.06
miR-19a-3p	0.05	0.06	0.07	-0.18	-0.20	-0.04	-0.06	0.02	-0.13	0.10	0.31	-0.04
miR-19b-3p	-0.01	-0.09	0.14	-0.20	-0.16	-0.07	-0.14	-0.05	-0.13	0.19	0.37*	0.07
miR-215-5p	-0.18	0.04	0.09	-0.04	-0.12	-0.08	0.02	0.06	-0.05	0.13	0.27	0.10
miR-29b-3p	0.08	0.30	-0.07	0.11	-0.15	0.15	0.30	0.37*	0.19	0.17	0.23	-0.19
miR-30e-5p	0.11	-0.12	0.10	-0.03	0.02	-0.09	-0.17	-0.07	-0.16	0.10	0.33	0.02
miR-320a	0.05	0.34*	-0.06	0.06	-0.33	-0.02	0.03	0.13	0.05	-0.21	-0.32	-0.12
miR-324-3p	-0.05	0.13	0.13	-0.01	-0.26	-0.02	-0.15	-0.21	-0.15	-0.04	0.46*	0.24
miR-335-5p	-0.16	0.24	0.19	-0.22	-0.14	-0.21	-0.05	-0.04	-0.01	-0.24	-0.01	-0.12
miR-365a-3p	0.19	0.11	0.02	0.14	0.50*	0.11	0.36*	0.48*	0.38*	0.07	-0.14	-0.20
miR-378a-5p	0.18	0.13	0.17	0.22	0.18	-0.19	0.16	0.15	0.00	0.30	0.05	-0.21
miR-532-5p	0.19	-0.10	0.14	-0.18	-0.13	0.04	-0.23	0.08	0.06	0.05	0.36*	0.15
miR-550a-3p	0.22	0.09	0.11	-0.02	-0.14	-0.01	0.02	0.12	0.12	0.10	-0.06	0.03
miR-7-5p	0.24	0.16	0.26	-0.23	-0.21	0.04	-0.13	0.18	0.08	0.10	0.12	0.09
miR-93-5p	-0.01	0.02	0.19	-0.13	-0.05	-0.22	0.00	-0.03	-0.07	0.16	0.40*	0.05

Figure 3. Correlation between microRNAs and markers of bone turnover and BMD. Spearman rank-based correlation was applied to the dataset comprising 19 microRNAs with differential expression in all three subgroups and various serological markers of bone turnover, age, and BMI. Positive and negative correlation coefficients are highlighted in red and blue, respectively. Significance was tested based on the null-hypothesis r = 0 and is highlighted with an asterisk (*) following the coefficient.

Recent literature suggests important functions for the observed miRNAs in the regulation of bone metabolism. miR-152-3p and miR-335-3p have both been reported to bind and down-regulate Dickkopf-1, a soluble inhibitor of the *Wnt signaling pathway* (29, 30). The expression of miR-30e-5p was shown to be down-regulated during osteogenic differentiation of MC3T3-L1 preosteoblasts, which results in the up-regulation of its direct target *Wnt signaling pathway* coreceptor, LRP-6 (31). Ding et al (32) further showed that ectopic expression of miR-30e-5p in MSCs and aortic smooth muscle cells resulted in the down-regulation of osteogenic gene panel. Conversely, down-regulation of miR-30e-5p in aortic smooth muscle cells enhanced calcification. miR-140–5p is known to be enriched in populations of undifferentiated MSCs from various tissue sources and to inhibit osteogenic differentiation by targeting BMP-2 (33). miR-550a was recently observed to inhibit osteogenic differentiation of adipose tissue derived MSCs (34). miR-324-3p and miR-19a/b-3p have not yet been described to directly affect bone remodeling. However, miR-324-3p and miR-19b-3p were correlated with aBMD at the lumbar spine in the present study.

miR-29b-3p was recently reported to regulate extracellular matrix formation via collagen type I expression (35). It is also known to down-regulate multiple genes that are involved in the regulation of osteoblast formation such as HDAC4, TGFβ3, ACVR2A, CTNNBIP1, DUSP2, and to be induced by TNFα/RANKL in osteoclast precursor cells to support the formation of mature osteoclasts (36). These results give evidence of an osteoblast dysfunction in patients with idiopathic fractures, as has been suggested previously (2). Overexpression of miR-365-3p can interfere with osteoclast formation in vitro (37) and in vivo (38). Supplemental Figure 6 provides an overview of the interactions between the here identified miRNAs and bone cells.

We observed that pretreatment of patients with anticatabolic therapy did not significantly alter the effect size of miRNA levels compared with matched controls. Nevertheless, the effect sizes observed for pretreatment vs no treatment suggest potential response of miRNA biomarkers to therapeutic intervention. Stronger effects in up-regulation and down-regulation of miRNAs, respectively, were found for pretreated patients. Due to the relatively low sample size and the small number of patients who received antiosteoporotic drugs, further analysis was not possible.

Based on the 2010 American Association of Clinical Endocrinologists Clinical Practice Guideline, BTMs may be used at baseline to identify patients with high bone turnover and can be used to monitor osteoporosis treatment (39). However, neither BTMs, nor aBMD nor clinical risk factors explained fracture occurrence in our study population. Patients with low-traumatic fractures showed significantly different miRNA signatures when compared with subjects without fractures. Nineteen miRNA were regulated in all patients with low-traumatic fractures, independent of age and sex. Several but not all miRNAs showed correlations to established BTMs or aBMD, suggesting additional information on bone metabolism. miR-29b-3p in particular has previously been reported as regulator of osteogenic differentiation and could serve as novel marker of bone turnover as a member of a miRNA signature.

Our study has several limitations. The comparison of BTMs was only performed between the groups of patients (premenopausal study arm [pre-MP], postmenopausal study arm [post-MP], MIO), but not with their healthy controls, given that BTMs were not analyzed in healthy subjects. The nonrandom selection of miRNA that were studied is another limitation of the present study.

Conclusion

Circulating miRNAs are promising biomarker candidates to assess the presence and progression of chronic multi-

factorial diseases: miRNAs are present in various biofluids, are robust against degradation due to long-term storage and freeze/thaw cycles, and their levels seem to exhibit low intra- and interindividual variation due to circadian rhythm or sex.

The miRNA signatures as found in the present study suggest new opportunities in the diagnosis of premenopausal and male idiopathic osteoporosis. Typical miRNA signatures could help to estimate bone fragility regardless of sex and age in future. Circulating miRNAs could serve as a clinical tool for fracture risk prediction.

Acknowledgments

The authors thank Tommy Vacca at Linz/Austria for proofreading. The authors also acknowledge the work of Dragana Simic and Patricia Gumbo from the VINFORCE study group and Annemarie Ferstl-Rohrbacher from the Medical University Graz/Austria.

Individual contributions included the following: R.K., M.H., and H.Red. for the study conception and design; R.K., R.D., F.P., and X.F. for the data collection; M.H., E.G., S.S., A.B., and P.H. for the data analysis; R.K., M.H., C.M., H.Red., H.Res., J.G., and A.F.-P. for the data interpretation; R.K., M.H., and J.G. for the drafting of the manuscript, the Figures, and the literature research; H.Red., H.Res., A.F.-P., and C.M. for the data interpretation and the extensive revision of the manuscript.

Address all correspondence and requests for reprints to: Dr Matthias Hackl, TAmiRNA GmbH, Muthgasse 18, 1190 Vienna, Austria. E-mail: matthias.hackl@tamirna.com.

This work was supported by the Austrian Society for Bone and Mineral Research (Project-Award 2014), by the Christian Doppler Gesellschaft, EU-FP7 Health Project FRAILOMIC 305483 and EU-FP7 Health Project SYBIL 602300.

Disclosure Summary: M.H. and J.G. are cofounders of TAmiRNA GmbH; M.H., E.G., and S.S. are employed by TAmiRNA GmbH. J.G. is a scientific advisor to TAmiRNA GmbH; M.H. and J.G. hold patents related to the use of circulating microRNAs as biomarkers for bone diseases. R.K., C.M., A.B., R.D., F.P., X.F., P.H., A.F.-P., H.Red., and H.Res. have nothing to declare.

References

1. **Gennari L, Bilezikian JP.** Idiopathic osteoporosis in men. *Curr Osteoporos Rep*. 2013;11(4):286–298.
2. **Patsch JM, Kohler T, Berzlanovich A, et al.** Trabecular bone microstructure and local gene expression in iliac crest biopsies of men with idiopathic osteoporosis. *J Bone Miner. Res*. 2011;26(7):1584–1592.
3. **Muschitz C, Kocijan R, Pahr D, et al.** Ibandronate increases sclerostin levels and bone strength in male patients with idiopathic osteoporosis. *Calcif Tissue Int*. 2015;96(6):477–489.
4. **Laroche M.** Heterogeneity of biological bone markers in idiopathic male osteoporosis. *Rheumatol Int*. 2012;32(7):2101–2104.
5. **Lapauw B, Vandewalle S, Taes Y, et al.** Serum sclerostin levels in men with idiopathic osteoporosis. *Eur J Endocrinol*. 2013;168(4):615–620.
6. **Siris ES, Miller PD, Barrett-Connor E, et al.** Identification and fracture outcomes of undiagnosed low bone mineral density in postmenopausal women: Results from the National Osteoporosis Risk Assessment. *JAMA*. 2001;286(22):2815–2822.
7. **Pillai RS, Bhattacharyya SN, Artus CG, et al.** Inhibition of translational initiation by Let-7 MicroRNA in human cells. *Science*. 2005;309(5740):1573–1576.
8. **Jing D, Hao J, Shen Y, et al.** The role of microRNAs in bone remodeling. *Int J Oral Sci* 2015;7(3):131–143.
9. **Olivieri F, Rippo MR, Procopio AD, Fazioli F.** Circulating inflamma-miRs in aging and age-related diseases. *Front Genet*. 2013;4:121.
10. **Sheinerman KS, Umansky SR.** Circulating cell-free microRNA as biomarkers for screening, diagnosis and monitoring of neurodegenerative diseases and other neurologic pathologies. *Front Cell Neurosci*. 2013;7:150.
11. **Li JY, Yong TY, Michael MZ, Gleadle JM.** Review: The role of microRNAs in kidney disease. *Nephrology (Carlton)*. 2010;15(6):599–608.
12. **Dhahbi JM, Spindler SR, Atamna H, Boffelli D, Mote P, Martin DIK.** 5′-YRNA fragments derived by processing of transcripts from specific YRNA genes and pseudogenes are abundant in human serum and plasma. *Physiol Genomics*. 2013;45(21):990–998.
13. **Huang X, Yuan T, Tschannen M, et al.** Characterization of human plasma-derived exosomal RNAs by deep sequencing. *BMC Genomics*. 2013;14:319.
14. **Turchinovich A, Weiz L, Burwinkel B.** Extracellular miRNAs: The mystery of their origin and function. *Trends Biochem Sci*. 2012;37(11):460–465.
15. **Weilner S, Schraml E, Wieser M, et al.** Secreted microvesicular miR-31 inhibits osteogenic differentiation of mesenchymal stem cells. *Aging Cell* 2016;15(4):744–754.
16. **Weilner S, Skalicky S, Salzer B, et al.** Differentially circulating miRNAs after recent osteoporotic fractures can influence osteogenic differentiation. *Bone*. 2015;79:43–51.
17. **Hackl M, Heilmeier U, Weilner S, Grillari J.** Circulating microRNAs as novel biomarkers for bone diseases—Complex signatures for multifactorial diseases? *Mol Cell Endocrinol*. 2016;432:83–95.
18. **Bessette L, Ste-Marie LG, Jean S, et al.** The care gap in diagnosis and treatment of women with a fragility fracture. *Osteoporos Int*. 2008;19(1):79–86.
19. **Blondal T, Jensby Nielsen S, et al.** Assessing sample and miRNA profile quality in serum and plasma or other biofluids. *Methods*. 2013;59(1):S1–S6.
20. **Andersen CL, Jensen JL, Ørntoft TF.** Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res*. 2004;64(15):5245–5250.
21. **Hind K, Oldroyd B, Truscott JG.** In vivo precision of the GE Lunar iDXA densitometer for the measurement of total-body, lumbar spine, and femoral bone mineral density in adults. *J Clin Densitom*. 2010;13(4):413–417.
22. **Metsalu T, Vilo J.** ClustVis: A web tool for visualizing clustering of multivariate data using Principal Component Analysis and heatmap. *Nucleic Acids Res*. 2015;43(W1):W566–W570.
23. **R Development Core Team.**R: A Language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing; 2016. Available at: http://www.R-project.org.
24. **Mooney C, Raoof R, El-Naggar H, et al.** High throughput qPCR expression profiling of circulating microRNAs reveals minimal sex- and sample timing-related variation in plasma of healthy volunteers. *PLoS One* 2015;10(12):e0145316.
25. **Cao Z, Moore BT, Wang Y, et al.** MiR-422a as a potential cellular microRNA biomarker for postmenopausal osteoporosis. *PLoS One*. 2014;9(5):e97098.

26. **Li H, Wang Z, Fu Q, Zhang J.** Plasma miRNA levels correlate with sensitivity to bone mineral density in postmenopausal osteoporosis patients. *Biomarkers*. 2014;19(7):553–556.
27. **Seeliger C, Karpinski K, Haug AT, et al.** Five freely circulating miRNAs and bone tissue miRNAs are associated with osteoporotic fractures. *J. Bone Miner Res*. 2014;29(8):1718–1728.
28. **Panach L, Mifsut D, Tarín JJ, Cano A, García-Pérez MÁ.** Serum circulating microRNAs as biomarkers of osteoporotic fracture. *Calcif Tissue Int*. 2015;97(5):495–505.
29. **Xu Y, Chen B, George SK, Liu B.** Downregulation of MicroRNA-152 contributes to high expression of DKK1 in multiple myeloma. *RNA Biol*. 2015;12(12):1314–1322.
30. **Zhang J, Tu Q, Bonewald LF, et al.** Effects of miR-335–5p in modulating osteogenic differentiation by specifically downregulating Wnt antagonist DKK1. *J Bone Miner Res*. 2011;26(8):1953–1963.
31. **Wang J, Guan X, Guo F, et al.** miR-30e reciprocally regulates the differentiation of adipocytes and osteoblasts by directly targeting low-density lipoprotein receptor-related protein 6. *Cell Death Dis*. 2013;4:e845.
32. **Ding W, Li J, Singh J, et al.** miR-30e targets IGF2-regulated osteogenesis in bone marrow-derived mesenchymal stem cells, aortic smooth muscle cells, and ApoE-/- mice. *Cardiovasc Res*. 2015; 106(1):131–142.
33. **Hwang S, Park SK, Lee HY, et al.** miR-140–5p suppresses BMP2-mediated osteogenesis in undifferentiated human mesenchymal stem cells. *FEBS Lett*. 2014;588(17):2957–2963.
34. **Heilmeier U, Hackl M, Skalicky S, et al.** Serum microRNAs are indicative of skeletal fractures in postmenopausal women with and without type 2 diabetes and influence osteogenic and adipogenic differentiation of adipose-tissue derived mesenchymal stem cells in vitro [published online ahead of print Jun 27, 2016]. *J Bone Miner Res*. doi:10.1002/jbmr.2897.
35. **Li Z, Hassan MQ, Jafferji M, et al.** Biological functions of miR-29b contribute to positive regulation of osteoblast differentiation. *J Biol Chem*. 2009;284(23):15676–15684.
36. **Franceschetti T, Kessler CB, Lee SK, Delany AM.** miR-29 promotes murine osteoclastogenesis by regulating osteoclast commitment and migration. *J Biol Chem*. 2013;288(46):33347–33360.
37. **Franceschetti T, Dole NS, Kessler CB, Lee SK, Delany AM.** Pathway analysis of microRNA expression profile during murine osteoclastogenesis. *PLoS One*. 2014;9(9):e107262.
38. **Li G, Bu J, Zhu Y, Xiao X, Liang Z, Zhang R.** Curcumin improves bone microarchitecture in glucocorticoid-induced secondary osteoporosis mice through the activation of microRNA-365 via regulating MMP-9. *Int J Clin Exp Pathol*. 2015;8(12):15684–15695.
39. **Adler RA, El-Hajj Fuleihan G, Bauer DC, et al.** Managing osteoporosis in patients on long-term bisphosphonate treatment: Report of a task force of the American Society for Bone and Mineral Research. *J Bone Miner Res*. 2016;31(1):16–35.