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Bone and muscle specific circulating microRNAs in postmenopausal women based on osteoporosis and sarcopenia status

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ABSTRACT

MicroRNAs (miRNAs) are short, non-coding RNA molecules that fine tune posttranscriptional protein expression. Aging is accompanied by progressive declines in muscle mass and strength, and in bone mineral density (BMD). Although miRNAs in pathology have been extensively studied, the role of circulating miRNAs (c-miRNAs) in osteoporosis and sarcopenia has to date not been well understood. The purpose of this study was to examine the difference in bone and muscle specific c-miRNAs in postmenopausal women based on their bone and muscle status, and to determine the associations between these specific c-miRNAs and muscle and bone variables. Seventy-five postmenopausal women aged 60 to 85 years old participated in this study. Body composition and BMD, functional performance tests (grip strength, gait speed, and countermovement jumps) were assessed. Levels of c-miRNAs (miR-1-3p, -21-5p, -23a-3p, -24-3p, -100-5p, -125b-5p, -133a-3p, -206) and bone turnover markers were analyzed. Statistically, there were no significant differences in specific c-miRNAs based on sarcopenia and osteoporosis status. However, fold changes of miR-21-5p (FC = 2.59) and -23a-3p (FC = 2.09) indicated upregulation and miR-125b-5p (FC = 0.46) indicated downregulation in the osteoporotic group compared to the non-osteoporotic group. The relative expression level of miR-125b-5p was significantly positively correlated with age ($p < 0.05$). The relative expression level of miR-21-5p was significantly negatively correlated with trochanter BMC ($p < 0.05$). Furthermore, the relative expression level of miR-23a-3p was significantly positively correlated with TRAP5b levels ($p < 0.05$). Although no statistical differences were found in target c-miRNAs based on muscle and bone status, our results indicate that there are biological differential expressions in some c-miRNAs between osteoporotic and non-osteoporotic individuals. Other circulating miRNAs need to be studied in the future.

1. Introduction

Fracture is a major contributor to health care costs and a societal burden as a result of the worldwide increase in the aging population [1]. Large cohort studies show that nearly one of two women and one of five men aged 50 years and older will have a fracture over the rest of their lifetime, and over two thirds of all fractures affect women over the age of 65 [2–4]. Currently, low bone mineral density (BMD) and osteoporosis are considered primary risk factors of fractures [5]. However, sarcopenia and other non-skeletal factors may also lead to the clinical consequence of fractures; for example, 90% of hip fractures occur after a simple fall [6].

Bone status and osteoporosis risk are assessed by BMD measurement by dual energy x-ray absorptiometry (DXA) and by bone turnover markers (BTMs) that reflect bone metabolic activity. Clinically, serum N-terminal propeptide of type I procollagen (PINP) and C-terminal cross-linking telopeptide of type I collagen (CTX-I) are the referent markers of bone formation and resorption, respectively, to predict the risk of fracture or monitor osteoporosis treatment [7]. PINP is a product of the cleavage of type I procollagen molecules, and CTX-I is derived from the breakdown of type I collagen. Another widely used bone resorption marker, tartrate-resistant acid phosphatase 5b (TRAP5b), is the enzyme representing the metabolic activity of osteoclasts [7]. During menopausal transition, levels of BTMs are elevated remarkably, particularly the bone resorption markers, indicating accelerated bone loss in postmenopausal women [8].

Since 2000, microRNAs (miRNAs) are recognized as a distinct class of biological regulators in human gene expression and the research on miRNAs is emerging [9]. MiRNAs are short non-coding RNA molecules that regulate posttranscriptional gene expression. MiRNAs are

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processed through primary miRNAs (pri-miRNA) by the enzyme, Drosha, to form precursor miRNAs (pre-miRNA), which are further processed via the enzyme, Dicer, to generate mature miRNAs [10]. The initial research on miRNAs has focused primarily at the tissue level, and is found to be related to diseases, such as cancer, cardiovascular diseases, and Alzheimer's diseases. Recent research has found some miRNAs, such as miR-1, -133, and -206, are abundant specifically in muscle, whereas some other miRNAs regulate osteogenesis and are associated with bone diseases, such as osteoporosis and arthritis [10–12]. Mature miRNAs are stable in human body fluids such as plasma [13,14]. This suggests that circulating miRNAs (c-miRNAs) may be used as potential biomarkers for diagnosis and treatment of diseases. Recently, certain c-miRNAs, such as miR-21 and -125b, were reported to be significantly upregulated in osteoporotic hip patients compared to controls [15,16]. However, the regulation of c-miRNAs in human body in various conditions, such as osteoporosis, fractures, aging muscles and exercise, is not yet fully understood.

Considering the role of miRNAs in regulating bone metabolism, it is reasonable to assume that bone turnover could be assessed by the measurement of bone-specific c-miRNAs that reflect bone resorption and formation. In addition, little is known regarding the role of skeletal muscle-specific c-miRNAs in sarcopenia in aging. Since that muscle-specific miRNAs regulate pathways, such as insulin-like growth factor-1 (IGF-1)/mammalian target of rapamycin (mTOR), it is possible that they may be associated with muscle loss in aging [17]. Finally, given that bone and muscles are connected in mechanotransduction and metabolic signaling, and both osteoporosis and sarcopenia are key contributors to fractures, it may be useful to include sarcopenia status in fracture risk model. Therefore, the primary purpose of this study was to examine the difference in bone and muscle specific c-miRNAs in postmenopausal women based on their bone and muscle status, and the secondary purpose was to determine the associations between these specific c-miRNAs and muscle and bone variables. We hypothesized that bone and muscle specific c-miRNAs would be upregulated in postmenopausal women with bone loss and muscle loss, respectively, and these specific c-miRNAs would be negatively associated with bone mass, bone strength, muscle mass, muscular strength and power.

2. Methods

2.1. Study participants

Community-dwelling postmenopausal women between 60 and 85 years of age ($n = 75$) were recruited for this study. A subset of participants ($n = 15$) were measured twice on bone measurements and functional performance tests. Prior to participation, participant signed written informed consent and HIPAA forms. The study was approved by the University of Oklahoma Health Sciences Center (OUHSC) Institutional Review Board (IRB#6971). Inclusion criteria were: women 60–85 years of age; independent living. Exclusion Criteria were: current smokers; women with diabetes; women with uncontrolled hypertension; women taking medications that affected bone metabolism, such as antidepressants, glucocorticoids; those had metal implants or joint replacement at hip or spine; women had recent fractures within 12 months; those had restrictions to perform functional performance or balance tests, such as myocardial infraction, congestive heart failure, strokes/back surgery within the past 6 months; women exceeded weight (158.8 kg) or height (1.93 m) limits of the bone densitometer.

2.2. Research design

Three visits were required in this study: consenting, blood pressure, and questionnaires (visit 1); blood draw and measurement of DXA and familiarization (visit 2); functional performance tests (handgrip strength, gait speed, jump test, balance) (visit 3). However, to assess reliability and consistency of our measures, a subset of 15 participants repeated the measurement of DXA and functional performance in an additional visit (visit 4). The fourth visit was 3–7 days after the third visit. A medical clearance form signed by the participant's physician was obtained prior to scheduling the following visits.

2.3. Questionnaires

Questionnaires were filled out by the participants to gather information regarding potential confounding variables that affect bone health and muscle performance. Health status questionnaire was used to identify whether the participant meet the inclusion and exclusion criteria for the study and to record medications taken by the participant. Menstrual history questionnaire was used to provide information about menstrual cycle and hormone replacement therapy history. Bone-specific physical activity questionnaire (BPAQ) was used to quantify exposure to bone loading physical activity throughout the lifespan [18]. Calcium Intake Questionnaire was used to estimate daily calcium intake from diet and supplements.

2.4. Anthropometric and blood pressure measurements

Height and weight were measured using a wall stadiometer (PAT #290237, Novel Products, Rockton, IL) and digital electronic scale (BWB-800, Tanita Corporation of America, Inc., Arlington Heights, IL). Resting blood pressure was measured by an automatic blood pressure monitor (Omron, Japan) on the left arm in the seated position. Participants with systolic blood pressure above 140 mm Hg or diastolic blood pressure above 90 mm Hg were considered hypertensive and thus excluded from this study.

2.5. Bone measurements

Dual energy X-ray absorptiometry (DXA) with enCORE software version 16 (Lunar Prodigy, GE Healthcare, Madison, MI) was used to measure and analyze areal bone mineral density (aBMD) of total body, AP lumbar spine (L1–L4), and dual proximal femur. Body composition of the whole body and regional areas, such as lean mass at arms and legs, were also obtained through the total DXA scan. A quality assurance test was performed at the beginning of each testing day prior to data collection. All DXA scans were assessed by the same technician. The CV% for BMD ranged from 1.0% to 1.8%, and CV% for body composition ranged from 1.2% to 2.1%.

2.6. Functional performance tests

Grip strength was measured using a handgrip dynamometer (Takei Scientific Instruments, Yashiroda, Japan) in seated position in both hands for three trials. Maximal handgrip strength was used in analyses. Gait speed was measured using an 8-meter straight path with participants walking at their usual pace for three trials. The average time of three repetitions was used to calculate the gait speed (distance/time). Muscle power was assessed by a jump test on a jump mat (Just Jump, Probotic, AL) with a Tendo FiTRODYNE power and speed analyzer (Tendo Sports Machines, Trencin, Slovak Republic). Participants were asked to do a countermovement vertical jump by crouching, then jumping with non-restricted arm motion, and then landing on the jump mat. A transfer belt was fastened around the waist of the participant, and trained spotters were standing on either side of the participant to help with balance, if needed. The average performance of the three trials was used in the data analysis.

The functional performance tests demonstrated good test-retest reliability in postmenopausal women: the intraclass correlation (ICC) ranged from 0.78 to 0.94 and the Pearson r ranged from 0.81 to 0.94. Sarcopenia status in postmenopausal women was determined based on the conventional definition as well as the criteria set by EWGSOP [19]. Conventional definition: skeletal muscle mass index (SMI) < -2 SD

Table 1
Candidate miRNAs that regulate cellular processes in muscle and bone.

MiRNA	Target(s)	Biological function
miR-1	PAX7, IGF1, etc.	+ myoblast differentiation
miR-133a	RUNX2	− osteoblast differentiation
	SLC39A1, etc.	+ myoblast differentiation
miR-206	CX43	− osteoblast differentiation
	PAX7, etc.	+ myoblast differentiation
miR-21	PDCD4, FASL	+ osteoblast differentiation and mineralization; + osteoclastogenesis and bone-resorbing activity
	EIF4E3, PDCD4	− apoptosis of muscle fiber [41]
miR-23a	RUNX2	− osteoblast differentiation
miR-24	RUNX2	− osteoblast differentiation
miR-100	BMPR2	− osteoblast differentiation
miR-125b	PDGF	+ osteocytes and chondrocytes differentiation; − osteoblast differentiation

+: promote; −: inhibit.

average reference population, that is, SMI < 5.5 kg/m^2 for women. EWGSOP criteria for women: gait speed < 0.8 m/s or grip strength < 20 kg, plus SMI < 5.5 kg/m^2.

2.7. Blood sampling and biochemical markers of bone turnover

A blood sample was collected in the early morning after overnight fasting. Blood samples were allowed to clot at room temperature for at least 30 min and then centrifuged at 1000*g* for 10 min at room temperature. Serum samples were aliquoted into microtubes, and immediately frozen at −80 °C until analyzed. Hemolysis was assessed via visual inspection of pink discoloration of serum samples. No samples had to be excluded due to hemolysis. Concentrations of bone resorption markers CTX-I and TRAP5b were measured in duplicate using the ELISA kits: CTX-I (Immunodiagnostic Systems, Gaithersburg, MD) and TRAP5b (Quidel, Athens, OH). The intra-assay CV% was 1.3–15.2%, and the inter-assay CV% was 1.3–7.0%.

Three muscle-specific miRNAs and five bone-specific miRNAs (miR-1-3p, -21-5p, -23a-3p, -24-3p, -100-5p, -125b-5p, -133a-3p, -206), were selected as representatives of those previously implicated underlying cellular processes (Table 1). Note that some of these miRNAs regulate several functions. For example, miR-21 has been reported to be associated with both osteoporosis and fractures [15] and sarcopenia in the elderly [20]. MiR-133a not only regulates myogenesis, but it is also a potential biomarker for osteoporosis [21].

2.8. RNA extraction and cDNA synthesis

Total RNA was extracted using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany). Serum samples were thawed at room temperature. 200 μL of serum was used for sample lysis by mixing with 1000 μL QIAzol Lysis Reagent, then 200 μL chloroform was added and phase separation was achieved by centrifugation for 15 min at 12,000*g* at 4 °C. Next, 650–750 μL of the upper aqueous phase was transferred to a new collection tube, which was further precipitated and purified on the QIAcube (Qiagen, Hilden, Germany). Finally, RNA was eluted in 15 μL RNase-free water, which yielded 13 μL of total RNA. RNA purity was determined using a DeNovix DS-11 Spectrophotometer (DeNovix, Wilmington, DE): A260/A280 ranged 1.9 to 2.1 indicating pure RNA. Extracted RNA samples were immediately frozen in a −80 °C freezer.

The extracted total RNA was transcribed to cDNA using the TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Foster City, CA). Total RNA and cDNA synthesis reagents were thawed to room temperature on ice. All miRNAs were amplified in a single reverse transcription (RT) reaction in four steps: poly(A) tailing reaction, adaptor ligation reaction, reverse transcription reaction, and miR-Amp reaction. In the last step, 45 μL of the miR-Amp Reaction Mix and 5 μL of the RT reaction were mixed in each new PCR tube, and universal cDNA reaction was performed in a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA) to amplify the cDNA molecules through the following steps: enzyme activation, denaturing and annealing cycles, and stop reaction. A total volume of 50 μL miR-Amp reaction product of each sample was frozen in −80 °C freezer immediately.

2.9. Quantification of miRNA expression

TaqMan Advanced miRNA assays (Applied Biosystems, Foster City, CA) and quantitative real-time Polymerase Chain Reaction (qPCR) were used to quantify the relative expression levels of selected c-miRNAs. Each 10 μL miR-Amp reaction was diluted with 180 μL 0.1× TE buffer (1:10) to prepare 200 μL of diluted cDNA template. 15 μL of PCR Reaction Mix was mixed with 5 μL of the diluted cDNA template in triplicate in a Fast Optical 96-well PCR Reaction Plate with Barcode (Applied Biosystems, Foster City, CA). Three wells of internal control (positive control) and another 3 wells of non-template control (negative control) were included at the end of each PCR reaction plate. Then the PCR reaction plate was loaded in the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA), and cDNA was amplified via fluorescently labeled TaqMan probes and primers through enzyme activation, denaturation and annealing/extension. MiR-16-5p, -93-5p, and -191-5p were selected as endogenous controls based on previous literature. MiR-16-5p and -93-5p were reported as the most stably expressed serum miRNAs in both cancer patients and healthy controls, whereas miR-191-5p and miR-93-5p were found the most stable miRNAs in both normal and tumor tissues [22–24]. The RQ Manager, version 1.2.1 (Applied Biosystems, Foster City, CA) was used to detect the C_t values to further analyze the real-time PCR results. The relative expression levels of target miRNAs were normalized to the normalization factor, which was calculated as the geometric mean of the three endogenous controls (miR-16-5p, -93-5p, -191-5p). Furthermore, fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method and log 2 transformation [25]. MiRNAs with Ct ≥37 were considered not expressed.

2.10. Statistical analysis

SPSS Statistics (SPSS Inc., Chicago, IL) was used to analyze data. The relative expression levels of c-miRNA were reported as mean ± standard error (SE), and the rest of descriptive data were reported as mean ± standard deviation (SD). Normality of the dependent variables was assessed using the Shapiro-Wilk tests. Variables of physical characteristics, bone and muscle, and functional performance were normally distributed, however, variables of miRNAs and BPAQ scores were not normally distributed. Osteoporosis was determined using aBMD T-scores at lumbar spine, femoral neck, or total hip according to WHO criteria (aBMD T-score ≤ −2.5), whereas sarcopenia was determined using conventional, as well as EWGSOP [19], definitions. Participants were further divided into non-osteoporotic/non-sarcopenic (NN, aBMD T-score > −1 with normal SMI), osteoporotic/osteopenic (OO, aBMD T-score ≤ −1 with normal SMI), sarcopenic (SP, aBMD T-score > −1, SMI ≤ 5.5 kg/m^2) and sarco-osteopenic (SOP, aBMD T-score ≤ −1, SMI ≤ 5.5 kg/m^2) groups. Osteoporosis (OP) is defined by aBMD T-score ≤ −2.5. However, there only one participant fell into the SP group, therefore, this group was excluded from ANOVA analysis. Chi-square analyses were used to examine the associations between the prevalence of osteoporosis and sarcopenia. One-way ANOVA was used to compare variables among NN, OO, and SOP groups. In order to minimize inflation of the Type I error, a Bonferroni correction was made to adjust the level of significance ($p \leq 0.05$/number of tests). Relative expression levels of target c-miRNAs (miR-1-3p, -21-5p, -23a-3p, -24-3p, -100-5p, -125b-5p, -133a-3p, -206) in osteoporosis group alone, SP, or OO and SOP groups were compared to that of the NN group. Two-tailed Mann-Whitney *U* tests were used to compare the relative expression levels of c-miRNAs between NN and osteoporosis groups, and NN and SP groups, whereas two-tailed Kruskal-Wallis tests

were used to compare the relative expression levels of c-miRNAs among NN, OP, and SOP groups. Spearman correlation analysis was used to test the relationships between the c-miRNAs and age, bone variables, muscle mass, muscular strength, and muscle power. The level of significance was set at $p \leq 0.05$.

3. Results

3.1. Bone and muscle classifications

The prevalence of sarcopenia based on the conventional definition ($n = 16$, 21.3%) was about three times of that based on the EWGSOP definition ($n = 6$, 8.0%). Osteopenia and osteoporosis were particularly common in this group of participants with a total percentage of 81% ($n = 61$). Because the sarcopenia sample size was much smaller based on the EWGSOP definition, sarcopenia based on the conventional definition was used for statistical analyses in this study. Altogether, 17.3% of participants (NN, $n = 13$) had normal muscle mass and aBMD, 61.3% of participants (OO, $n = 46$) had osteoporosis/osteopenia (low bone mass) with normal muscle mass, 20% of participants (SOP, $n = 15$) had both sarcopenia and osteopenia (sarco-osteopenia), whereas only one participant (SP, $n = 1$, 1.3%) who had been on hormone replacement therapy (HRT) for over 45 years had sarcopenia with normal aBMD. Therefore, the statistical analysis of comparisons was performed only in the other three groups (NN, OO, and SOP).

3.2. Participant characteristics

The characteristics of NN, OO, SOP and SP groups are shown in Table 2. One to seven participants of each group were currently taking HRT for at least 6 months. There were no significant differences in age, height, calcium intake, BPAQ scores, or self-reported HRT history in NN, OO, and SOP groups. However, the SOP group had significantly lower body weight, muscle mass (BFLBM), and appendicular skeletal muscle mass (ASM) than the NN and OO groups. Table 3 shows the functional performance of the four groups of participants. Compared to the NN group, the SOP group had significantly lower grip strength ($p = 0.008$), jump power (approximate 20%) than NN and OO groups ($p = 0.044$ and $p = 0.003$, respectively). However, there were no significant differences in gait speed, jump height, jump velocity, or relative jump power among groups. Table 4 shows the areal bone mineral density (aBMD) for the total body, lumbar spine, and dual femur sites in the four groups of participants. Overall, the OO and SOP groups had significantly lower aBMD than the NN group ($p < 0.01$). Table 5 shows the concentrations of bone resorption markers CTX-I and TRAP5b in the four groups. No significant differences in BTMs were found among groups.

Table 3
Functional performance of the participants.

Variable	Group			
	NN ($n = 13$)	OO ($n = 46$)	SOP ($n = 15$)	SP ($n = 1$)
Grip strength (kg)	26.7 ± 4.6	24.5 ± 3.9	21.8 ± 4.9**	13.1
Gait speed (m/s)	1.28 ± 0.17	1.30 ± 0.19	1.31 ± 0.18	1.24
JHt (inch)	7.6 ± 1.8	7.2 ± 2.2	7.9 ± 2.0	5.73
JVel (m/s)	0.86 ± 0.17	0.89 ± 0.15	0.85 ± 0.12	0.81
JP (W)	600 ± 132.0	608.2 ± 125.5	485.0 ± 95.3*††	497.67
Relative JP (W/kg)	8.46 ± 1.72	8.76 ± 1.47	8.32 ± 1.18	8.13

Mean ± SD; NN: non-osteoporotic/non-sarcopenic; OO: osteoporotic/osteopenic; SP: sarcopenic; SOP: sarco-osteopenic. JHt: jump height; JVel: jump velocity; JP: jump power.
* $p < 0.05$, ** $p < 0.01$ significant difference between NN and SOP groups.
†† $p < 0.01$ significant difference between OO and SOP groups.

3.3. Expression of circulating miRNAs (c-miRNAs)

MiRNAs with Ct ≥ 37 were considered not expressed since these expression levels indicated large variability [16]. As a result, three samples were not expressed in 3 endogenous controls (miR-16-5p, -93-5p, -191-5p), another three sample were not expressed in 2 endogenous controls (miR-93-5p, -191-5p), and six samples were not expressed in 1 endogenous control (3 of miR-93-5p; 3 of miR-191-5p). In total, twelve samples were excluded from miRNA analysis due to the lack of quality normalization factors from endogenous controls, thus 63 samples were included in miRNA analysis. Only about a third of the 63 samples expressed circulating miR-1-3p ($n = 23$), -133a-3p ($n = 14$), -100-5p ($n = 24$) and none of the samples expressed circulating miR-206. Therefore, the relative expression levels of the rest of the target miRNAs (miR-21-5p, -23a-3p, -24-3p, -125b-5p) between the 4 groups, which are all bone-specific miRNAs, are reported in Supplementary Table 1 and Fig. 1(A–D). Biologically, fold change of > 2 (corresponding to a $\log_2$-fold change of 1) is considered upregulation, whereas fold change

Table 2
Physical characteristics of the participants.

Variable	Group			
	NN ($n = 13$)	OO ($n = 46$)	SOP ($n = 15$)	SP ($n = 1$)
Age (year)	68.9 ± 6.5	69.6 ± 5.6	68.9 ± 5.2	85.8
Height (cm)	165.3 ± 5.9	162.0 ± 6.3	163.4 ± 6.1	161.5
Weight (kg)	71.5 ± 11.9	70.0 ± 11.6	58.1 ± 6.6**††	61.2
Ca^{2+} (mg/day)	1730.8 ± 800.9	1486.8 ± 677.8	1630.6 ± 669.3	487.9
BPAQ past	72.5 ± 58.0	81.7 ± 62.4	130.0 ± 140.9	4.8
BPAQ current	4.9 ± 6.2	3.6 ± 5.0	4.8 ± 6.0	0.3
BPAQ total	38.7 ± 29.2	42.6 ± 31.9	67.4 ± 71.1	2.5
HRT Users (*n*/%)	5/38.5%	7/15.2%	6/40%	1/100%
HRT Time (year)	19.0 ± 15.0	13.4 ± 10.4	17.3 ± 7.4	45
% Body fat	39.8 ± 6.7	40.8 ± 7.0	37.7 ± 5.4	42.0
BFLBM (kg)	39.6 ± 3.6	38.7 ± 4.0	33.4 ± 2.0***†††	33.7
FM (kg)	28.8 ± 9.1	28.7 ± 9.3	21.9 ± 5.3†	26.0
ASM (kg)	16.8 ± 1.5	16.7 ± 2.1	13.7 ± 1.3***†††	13.5

Mean ± SD; NN: non-osteoporotic/non-sarcopenic; OO: osteoporotic/osteopenic; SP: sarcopenic; SOP: sarco-osteopenic. Ca^{2+}: calcium intake; BPAQ: bone-specific physical activity questionnaire; HRT Users (*n*/%): number and percentage of participants in each group that were current taking HRT; HRT Time: self-reported duration of current use of HRT; BFLBM: bone free lean body mass; FM: fat mass; ASM: appendicular skeletal mass.
** $p < 0.01$, *** $p < 0.001$ significant difference between NN and SOP groups.
† $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ significant difference between OO and SOP groups.

Table 4
Areal bone mineral density measured by DXA.

Variable	Group			
	NN (n = 13)	OO (n = 46)	SOP (n = 15)	SP (n = 1)
Total body	1.199 ± 0.057	1.110 ± 0.097$^{\#\#}$	1.089 ± 0.104**	1.203
Lumbar spine	1.264 ± 0.154	1.073 ± 0.163$^{\#\#}$	1.044 ± 0.140**	1.339
Left FN	0.976 ± 0.065	0.835 ± 0.083$^{\#\#\#}$	0.796 ± 0.098***	0.948
Right FN	0.969 ± 0.064	0.836 ± 0.074$^{\#\#\#}$	0.804 ± 0.088***	0.923
Left troch	0.814 ± 0.068	0.717 ± 0.095$^{\#\#}$	0.652 ± 0.109***	0.801
Right troch	0.799 ± 0.065	0.707 ± 0.088$^{\#\#}$	0.660 ± 0.115***	0.784
Left total hip	1.008 ± 0.058	0.881 ± 0.091$^{\#\#\#}$	0.828 ± 0.104***	0.992
Right total hip	0.990 ± 0.060	0.872 ± 0.087$^{\#\#\#}$	0.832 ± 0.103***	0.970

g/cm^2; Mean ± SD; NN: non-osteoporotic/non-sarcopenic; OO: osteoporotic/osteopenic; SP: sarcopenic; SOP: sarco-osteopenic. FN: Femoral Neck; Troch: Trochanter.
$^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ significant difference between NN and OO groups.
$^{**}p < 0.01$, $^{***}p < 0.001$ significant difference between NN and SOP groups.

Table 5
Serum bone turnover marker concentrations.

Variable	Group			
	NN (n = 13)	OO (n = 46)	SOP (n = 15)	SP (n = 1)
CTX-I (ng/mL)	0.24 ± 0.17	0.36 ± 0.20	0.33 ± 0.21	0.37
TRAP5b (U/L)	2.75 ± 1.29	3.53 ± 1.49	3.44 ± 2.01	2.84
C/T Ratio	0.098 ± 0.078	0.113 ± 0.068	0.101 ± 0.049	0.132

Mean ± SD; NN: non-osteoporotic/non-sarcopenic; OO: osteoporotic/osteopenic; SP: sarcopenic; SOP: sarco-osteopenic. C/T Ratio: CTX-I/TRAP5b ratio.

of lower than 0.5 (corresponding to a $\log_2$fold change of −1) is considered as downregulation [26]. In our study, fold changes ranged from 0.83 to 1.81, and no significant differences were found in the relative expression levels of these miRNAs (miR-21-5p, -23a-3p, -24-3p, -125b-5p) among NN, OO, and SOP groups.

Participants were further compared based on muscle status and bone status separately. When comparing the SP and non-sarcopenic (NSP) groups, no significant differences were observed in the relative expression levels of c-miRNAs (miR-21-5p, -23a-3p, -24-3p, -125b-5p) between the two groups and fold changes ranged from 0.86 to 1.66 (Table 6). Similarly, no significant differences were found in the relative expression levels of c-miRNAs (miR-21-5p, -23a-3p, -24-3p, -125b-5p) between non-osteoporotic (NOP) and osteoporosis groups

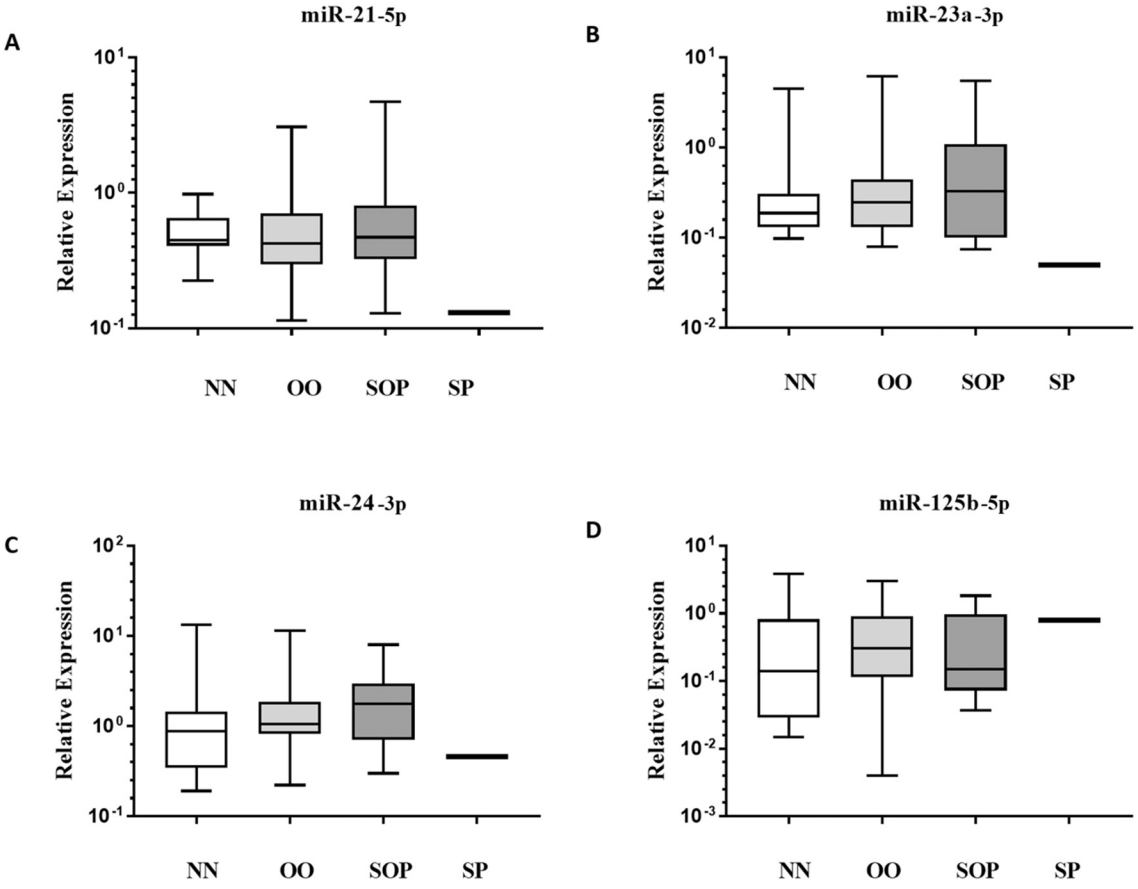

Fig. 1. Relative expression levels of miR-21-5p (A), -23a-3p (B), -24-3p (C), and -125b-5p (D) based on muscle and bone status (Mean ± SE).
NN: non-osteoporotic/non-sarcopenic; OO: osteoporotic/osteopenic; SP: sarcopenic; SOP: sarco-osteopenic.

Table 6
Relative expression levels of miRNAs in sarcopenia.

miRNA	Relative expression		Fold change	p
	NSP (n = 51)	SP (n = 12)		
miR-21-5p (n = 62)	0.63 ± 0.08 (n = 51)	0.85 ± 0.40 (n = 11)	1.35	NS
miR-23a-3p (n = 62)	0.58 ± 0.18 (n = 49)	0.97 ± 0.45 (n = 12)	1.66	NS
miR-24-3p (n = 62)	1.64 ± 0.34 (n = 51)	2.14 ± 0.69 (n = 11)	1.30	NS
miR-125b-5p (n = 62)	0.64 ± 0.13 (n = 44)	0.55 ± 0.19 (n = 11)	0.86	NS

Mean ± SE; NSP: non-sarcopenic; SP: sarcopenic. NS: no significant difference.

Table 7
Relative expression levels of miRNAs in osteoporosis.

miRNA	Relative expression		Fold change	p
	NOP (n = 55)	OP (n = 8)		
miR-21-5p (n = 62)	0.56 ± 0.06 (n = 54)	1.44 ± 0.57 (n = 8)	2.59	NS
miR-23a-3p (n = 61)	0.59 ± 0.17 (n = 54)	1.22 ± 0.71 (n = 7)	2.09	NS
miR-24-3p (n = 62)	2.21 ± 0.58 (n = 54)	1.72 ± 0.45 (n = 8)	0.99	NS
miR-125b-5p (n = 55)	0.67 ± 0.12 (n = 48)	0.31 ± 0.10 (n = 7)	0.46	NS

Mean ± SE; NOP: non-osteoporosis; OP: osteoporosis. NS: no significant difference.

(Table 7). However, fold changes of miR-21-5p (FC = 2.59) and miR-23a-3p (FC = 2.09) indicated upregulation in osteoporosis compared to NOP, whereas fold change of miR-125b-5p (FC = 0.46) indicated downregulation in osteoporosis compared to NOP.

Supplementary Table 2 compares the relative expression levels of miRNAs between HRT current users and non-users. Since there was a sufficient number of expressed miR-1-3p and miR-100-5p samples in HRT users, comparisons were made in miR-1-3p and -100-5p as well. No significant differences in relative expression levels were found between the current HRT users and non-users groups. Fold changes ranged from 0.23 to 1.57, indicating downregulation of miR-1-3p (FC = 0.23) in the HRT users compared to the non-users.

We examined characteristics of participants based on expression and non-expression of c-miRNAs. Interestingly, those who expressed miR-100-5p had significantly higher age, and calcium intake but significantly lower height, jump height, total aBMC, BPAQ scores (total and past) compared to participants who did not expressed miR-100-5p (Supplementary Table 3). Similarly, women who expressed miR-133a-3p were significantly older and had significantly lower jump height, total aBMD, BPAQ scores (past and total) compared to those who did not expressed miR-133a-3p (Supplementary Table 4).

3.4. C-miRNA correlations

The relative expression level of circulating miR-125b-5p was significantly positively correlated with age (r = 0.334, p = 0.012) (Supplementary Fig. 1). Spearman correlations showed that the relative expression levels of miR-21-5p were significantly negatively correlated to the left trochanter BMC (r = −0.252, p = 0.048) (Supplementary Fig. 2), and right trochanter BMC (r = −0.294, p = 0.020) (Supplementary Fig. 3). There also was a trend for a significant correlation between the relative expression of miR-21-5p and lumbar spine aBMD (r = −0.249, p = 0.051). Furthermore, the relative expression level of miR-23a-3p was found to be significantly positively correlated to the level of TRAP5b (r = 0.259, p = 0.044) (Supplementary Fig. 4). The relative expression level of miR-133a-3p was positively correlated with total body BMC (r = 0.594, p = 0.025), although only 14 samples were expressed in circulating miR-133a-3p. However, no significant correlations were found between other miRNAs (miR-24-3p, -100-5p, -125b-5p) and bone variables.

The relative expression level of miR-125b-5p was significantly positively correlated with jump velocity (r = 0.263, p = 0.05) (Supplementary Fig. 5) and relative jump power (r = 0.294, p = 0.028) (Supplementary Fig. 6). However, none of the muscle-specific circulating miRNAs (miR-1-3p, -133a-3p) were correlated with muscle mass, muscle strength, or muscle power.

4. Discussion

To our knowledge, this is the first study examining c-miRNAs in sarcopenia and sarco-osteopenia in postmenopausal women. Although no statistical significant differences in circulating bone and muscle-specific miRNAs were found in this study, SOP individuals were found at the greatest risk of low muscle mass and poor functional performance (grip strength) compared to NN and OO individuals. Circulating miR-21-5p level was negatively associated with trochanter BMC, whereas circulating miR-23a-3p level was positively associated with bone resorption marker, TRAP5b. In addition, circulating miR-125b-5p level was significantly positively associated with age, jump velocity and relative jump power. These relationships are in line with the regulatory function of these miRNAs on bone homeostasis genes, except for the positive associations between miR-125b-5p and jump velocity and relative jump power.

In this study, the prevalence of osteoporosis was 13% in postmenopausal Caucasian women aged 60 to 85 years, which is lower than the prevalence of previous study of approximately 21.6% in women in their 60s, 38.5% in their 70s, and as high as 70% in women over age 80 years [27]. The discrepancy in findings was probably due to having relative healthy participants in our study, and our sample size was much smaller than this previous study. The new definition of sarcopenia by the EWGSOP combines low muscle mass and poor functional performance. For example, the prevalence of sarcopenia in this study was reduced to 8% based on the EWGSOP definition. One of the challenges in sarcopenia research is the lack of consensus about the operational definition of sarcopenia and the evaluation of muscle strength and function among consensus groups and studies.

We did not find significant statistical differences in levels of c-miRNAs based on osteoporosis and sarcopenia statuses. However, fold changes of miR-21-5p (FC = 2.59) and miR-23a-3p (FC = 2.09) indicated upregulation in the osteoporosis group, whereas fold change of miR-125b-5p (FC = 0.46) indicated downregulation in the osteoporosis group. Seeliger et al. [15] found that five miRNAs were significantly upregulated in osteoporotic hip fracture patients (n = 10) in both serum and bone tissue compared to non-osteoporotic controls (n = 10), including miR-21-5p, -23a-3p, -24-3p, -100-5p, and -125b-5p. Panach et al. [16] found three c-miRNAs, miR-122-5p, -125b-5p, and -21-5p, that were significantly upregulated in osteoporotic hip fracture patients (n = 8) compared to osteoarthritic controls (n = 5). The discrepancy in findings is probably because our osteoporotic participants were not fracture patients, and they had no recent fractures within the previous 12 months. Levels of specific c-miRNAs between osteoporotic participants with and without fractures compared to non-osteoporotic controls might be different.

Interestingly, we found that the relative expression level of miR-21-5p was significantly negatively correlated with the trochanter BMC. Also, there was a trend for significant association between expression of miR-21-5p and lumbar spine aBMD. So far, miR-21-5p has been one of the most studied miRNAs in bone diseases. Particularly, circulating miR-21-5p has been consistently found to be upregulated in patients with osteoporotic fractures [15,16]. Panach et al. [16] found that the level of miR-21-5p was correlated with the levels of the bone resorption

marker, CTX-I ($r = 0.76$). MiR-21 regulates both osteoblast and osteoclast activities, and it is highly expressed in osteoclast precursors during osteoclastogenesis. One of the transcription factors, c-FOS, triggers miR-21 transcription. Meanwhile, miR-21 inhibits PDCD4 (programmed cell death protein 4) levels, which inhibits c-FOS. Therefore, a positive feedback loop of c-FOS/miR-21/PDCD4 is formed, which promotes RANKL-induced osteoclastogenesis or bone resorption activity [28]. On the other hand, estrogen inhibits osteoclastogenesis, and recent research has shown that this inhibitory effect is mediated by miR-21 [29]. In fact, miR-21 targets on Fas Ligand (FASL) gene, which induces osteoclast apoptosis [29]. Estrogen deficiency in postmenopausal women upregulates miR-21, which decreases the transcription of FASL. As a result, it inhibits osteoclast apoptosis and promotes bone resorption activity. This may explain the negative relationship between circulating miR-21-5p and bone variables in our findings.

In this study, we found a low positive correlation ($r = 0.259$) between the relative expression level of miR-23a-3p and the level of TRAP5b. TRAP5b, a bone resorption marker, is an enzyme released into the circulation during osteoclast activity. Research has shown that miR-23a targets RUNX2 pathway in mice, which further inhibits the differentiation of osteoblasts [30]. Therefore, high levels of miR-23a inhibit bone formation, and the net imbalance favors bone resorption. Similarly, high levels of TRAP5b indicate a greater number of osteoclasts, which favors bone resorption. The positive relationship between miR-23a-3p and TRAP5b found in this study may be attributed to their regulatory roles on bone resorption.

We found that the relative expression level of miR-125b-5p was significantly positively associated with jump velocity and relative jump power. To date, only a few studies have investigated the relationship between c-miRNAs and muscle performance, and most of them focused on acute exercise responses [31,32]. Banzet et al. [31] reported that the levels of circulating miR-1, -133a, -133b and -208b were significantly increased 6 h after 30-min downhill walking compared to baseline, whereas no significant changes were observed after 30-min uphill walking in healthy young men ($n = 9$). This result suggested that exercise responses of miRNAs were related to exercise mode, with no changes in concentric exercises but significant decreases in eccentric exercise. Gomes et al. [32] measured c-miRNA levels (miR-1, -133a, and -206) in recreational runners ($n = 5$) before and after half marathon, and found that circulating miR-1 (FC = 1.3), -133a (FC = 1.2), and -206 (FC = 4.3) were significantly upregulated after a half marathon run. MiR-206 is primarily expressed in slow-twitch fibers, and the upregulation of miR-206 reflected the predominance of slow-twitch fibers during half marathon.

An unexpected finding in our study was that muscle-specific circulating miRNAs, miR-1-3p, -133a-3p, and -206 were not expressed well in this group of postmenopausal women. Most of the studies examining muscle-specific miRNAs have been conducted at the tissue level, in younger adults, or during exercise conditions, and there are different characteristics between tissue-specific miRNAs and circulating miRNAs, in young and older populations, at rest and during exercise [33]. We focused on older women because this population has one of the highest risks of osteoporosis and sarcopenia. Previous research has shown that miRNA expressions are associated with age [34,35]. In this study, we found that miR-125b-5p was significantly positively correlated with age ($r = 0.334$, $p = 0.012$). Furthermore, miRNA responses are altered by age. Drummond et al. [36] found that miR-1 was reduced in young (mean 29 years) but not in older men (mean 70 years) following acute resistance exercise and essential amino acids ingestion, indicating that aging results in a dysregulated miRNA response after an anabolic stimulus. Rivas et al. [37] found that resistance exercise induced changes in skeletal muscle miRNA expression were only observed in the younger group but not older adults. They further confirmed that circulating miRNAs were upregulated in younger men but downregulated in older men in response to resistance exercise.

There are several limitations to this study. First, only a small number of samples of circulating miR-1-3p ($n = 23$), -133a-3p ($n = 14$), miR-100-5p ($n = 24$) and none of circulating miR-206 were expressed. MiR-1-3p, -133a-3p, and -206 are all muscle-specific miRNAs; thus, we were unable to examine the relationships between muscle-specific miRNAs and sarcopenia. Second, there were only 10 participants in the osteoporotic group compared to 6–7 times as many participants in the control group, which limited our statistical power. We examined only 8 targeted miRNAs associated with muscle and bone in the literature because of cost, thus, there may be other miRNAs not studied here that may be better indicators of osteoporosis and sarcopenia status. In addition, serum sample hemolysis was assessed via simple visual inspection, which is not the most sensitive method to detect hemolysis [38]. Moreover, although participants in this study were relatively healthy, there were a couple of cancer survivors and individuals with rheumatoid arthritis. Diseases conditions, such as degenerative diseases, malignancies, and autoimmune diseases, might alter miRNA expression. Last, their hormone status was not examined as some of them were currently taking HRT. More recently, there is an increasing interest in c-miRNAs responses to exercise, and research has indicated that some c-miRNAs were altered after exercise and correlated with fitness parameters [39].

There are challenges in studying miRNAs as biomarkers. First, no reference values of miRNA levels have been established in healthy populations or older adults. Second, most miRNA expression data are relative expression values, and there is no standard normalization method since the results are highly dependent on the endogenous controls chosen, the technology platform and its technical variation. Although miR-16-5p, -93-5p, and -191-5p were used as the endogenous controls in this study, as they were reported as the mostly stable expressed reference genes, a recent study found that miR-93-5p was significantly correlated with lumbar spine aBMD ($r = 0.40$) [40]. Adding spike-in controls to RNA extraction and PCR steps would be a better method to normalize miRNAs. Third, there are over 2800 miRNAs that have been identified in humans so far, and each miRNA may target on multiple genes. Therefore, it is may be better to use of a combination of several miRNAs rather than a single miRNA as disease biomarkers.

5. Conclusion

Though this study did not determine that these specific circulating miRNAs were biomarkers of osteoporosis and sarcopenia, we found that circulating miR-21 was significantly negatively correlated with some bone variables, and circulating miR-125b was significantly positively correlated with age, which gives us possible ideas to further explore characteristics of circulating miRNAs in a variety of populations. In addition, we found that the sarco-osteopenic individuals were at the greatest risk of poor functional performance. Therefore, sarco-osteopenia individuals should be the next focus in fall and fracture prevention.

The measurement of serum miRNAs is novel, and it reflects the regulation of gene expression, providing the underlying cellular and molecular process involved in bone turnover and muscle aging. MiRNA precursors (pri-miRNA, pre-miRNA) and target genes need to be studied to better understand the functions of target miRNAs. Other target miRNAs are needed to get a comprehensive understanding, and miRNA microarray is a better tool to analyze a large number of circulating miRNAs in one sample. For example, studies indicated that the miR-29 family (miR-29a, -29b, -29c) is a key mediator of osteoclast differentiation, and miR-31 is highly upregulated during RANKL-induced osteoclastogenesis [28]. Clinical populations, such as osteoporotic facture patients, as well as exercise responses in healthy populations, are promising directions to study miRNA profiles. Exercise interventions may be a better approach to study muscle-specific circulating miRNAs (miR-1, -133a, -206) in aging, since most of them were not expressed at rest in postmenopausal women in our study but changed in response to

exercise in healthy young men [31].

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2018.11.001.

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