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BONE AND MUSCLE SPECIFIC CIRCULATING MICRORNAS IN POSTMENOPAUSAL WOMEN BASED ON OSTEOPOROSIS AND SARCOPENIA STATUS

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BONE AND MUSCLE SPECIFIC CIRCULATING MICRORNAS IN POSTMENOPAUSAL WOMEN BASED ON OSTEOPOROSIS AND SARCOPENIA STATUS

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF HEALTH AND EXERCISE SCIENCE

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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 studied. **Purposes:** 1) To identify specific c-miRNAs that are associated with bone and muscle status in postmenopausal women and to evaluate the use of these c-miRNAs as biomarkers of osteoporosis and sarcopenia; and 2) to determine the associations between specific c-miRNAs and muscle and bone variables. **Methods:** Seventy-five postmenopausal women aged 60 to 85 years old participated in this study. Body composition and areal BMD (aBMD) were measured by DXA. Volumetric BMD (vBMD) and bone strength were measured by pQCT. Muscle performance tests, including grip strength, gait speed, and countermovement jumps, were assessed. Bone status was classified based on aBMD T-scores: Osteopenia ($-2.5 \leq$ aBMD T-score ≤ -1) and osteoporosis (aBMD T-score ≤ -2.5). Two sets of criteria were used to classify sarcopenia status: 1) skeletal muscle mass index (SMI) < 5.5 kg/m^2; and 2) SMI < 5.5 kg/m^2 and low muscle strength (grip strength < 20 kg) or low gait speed (<0.8 m/s). Levels of c-miRNAs (miR-1, -21, -23a, -24, -100, -125b, -133a, -206) were analyzed using real-time PCR, and bone turnover markers were analyzed by enzyme-linked immunosorbent assay (ELISA). **Results:** There was no significant association between sarcopenia and osteopenia status in postmenopausal women. The sarco-osteopenia group had significantly lower body weight, jump power, and muscle CSA at 66% of the tibia than the normal and

osteopenia groups ($p < 0.05$). Statistically, there were no significant differences in specific c-miRNAs based on sarcopenia and osteoporosis status. However, fold changes of miR-21 (FC=2.59) and -23a (FC=2.09) indicated upregulation and miR-125b (FC=0.46) indicated downregulation. The relative expression level of miR-125b was significantly negatively correlated with age ($p < 0.05$). The relative expression level of miR-21 was significantly negatively correlated with trochanter BMC and cortical vBMD at tibia 38% site ($p < 0.05$). Furthermore, the relative expression level of miR-23a was significantly positively correlated with TRAP5b levels ($p < 0.05$). **Conclusion**: There were no statistical differences in target circulating miRNAs (miR-1, -21, -23a, -24, -100, -125b, -133a, -206) based on bone and muscle status in postmenopausal women. However, fold changes of circulating miR-21, -23a, -125b indicated biological differential expression. Other circulating miRNAs need to be studied in the future.

Chapter 1 Introduction

Medical, social, and economic advances over diseases have dramatically increased life expectancy in the last century. Life expectancy in the U.S. now approaches 79 years of age, and in several other countries has exceeded 80 yr (89). As people are living longer and healthier, the entire society shifts towards an older population. The population of individuals over the age of 65 is projected to increase from 524 million in 2010 to approximately 1 billion by 2030 and 1.5 billion in 2050 (89). In the U.S., as baby boomers turn 65 years old by the year 2032, there will be more people over the age of 65 than children under the age of 15 (54). The term "oldest old" is defined as people 85 years and older, currently accounting for 8% of all older adults (≥ 65 yr) in the world and 14% of the older population (≥ 65 yr) in the U.S. (89). The oldest old now is growing fast in many countries, and is projected to increase at least 3 fold from 2010 to 2050 globally (89).

Global aging presents challenges, such as increased prevalence of chronic diseases and disability over time. Muscle fiber number begins to decrease after the age of middle 20's, leading to approximately 50% decline in skeletal muscle mass from ages 20 to 80 (39). In the process of aging, bone mineral content is lost gradually, and bone loss is accelerated during the menopausal period in women, with a loss of about 20-30% trabecular bone and 5-10% cortical bone (67). The age-related muscle loss is defined as sarcopenia, and bone loss is osteopenia or osteoporosis. The National Osteoporosis Foundation in 2014 estimated that over 10 million Americans had osteoporosis and an additional 43 million had osteopenia, with the majority of patients being older adults (22). Both sarcopenia and osteoporosis are associated with functional

impairment and disability or overall frailty, which decreases the quality of life and requires long-term care (90).

Currently, bone mineral density (BMD) measured by dual energy x-ray absorptiometry (DXA) is the gold standard for osteoporosis diagnosis based on the definition by World Health Organization (WHO). BMD decreases progressively with aging, and it is well documented that a one standard deviation (SD) decrease in BMD is associated with 1.5-2.6 fold increase of risk of fracture (77). The process of bone metabolism is called bone turnover and it consists of two opposite activities: bone formation and bone resorption. Bone turnover markers (BTMs) are enzymes (e.g. acid phosphatase) reflecting bone metabolic activity or bone matrix degradation products released into the circulation during bone resorption (76). Clinically, serum N-terminal propeptide of type I procollagen (PINP) and C-terminal cross-linking telopeptide of type I collagen (CTX) are proposed to be the referent markers of bone formation and resorption, respectively, to predict the risk of fracture or monitor osteoporosis treatment (131). PINP is a product of the cleavage of type I procollagen molecules, and CTX is derived from the breakdown of type I collagen (111). Another widely used bone resorption maker, tartrate-resistant acid phosphatase 5b (TRAP5b), is the enzyme representing the metabolic activity of osteoclasts. During menopausal transition, levels of BTMs are elevated remarkably, particularly the bone resorption markers, indicating accelerated bone loss in postmenopausal women (111).

Fracture risk increases exponentially with age. Fragility fracture is a major contributor to health care costs and the societal burden as worldwide increase of aging population. 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 (25, 34, 130). Vertebral fracture causes back pain and reduces physical function, with prevalence of 25-50% in women aged over 50, although only 30% of them are clinically recognized (34). Hip fracture is common and known to be associated with increased mortality and disability. In the U.S., the lifetime risk of hip fracture is estimated to be 17% for Caucasian women and 6% for Caucasian men (25). Current prevention of fractures in the elderly mainly depends on the diagnosis of osteoporosis (88). However, sarcopenia, poor balance and other non-skeletal factors such as falls, for example, 90% of hip fractures occur after a simple fall, increase the risk of fracture with advanced aging, which may also lead to the clinical consequence of fractures (61).

MicroRNAs (miRNAs), short non-coding RNA molecules that regulate posttranscriptional gene expression, were discovered two decades ago with over 2,800 miRNAs in human having been identified to date. 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 (74, 84, 117). Mature miRNAs are found to be stable in human body fluids such as plasma, and they protect themselves from cleavage by endogenous ribonuclease (RNase) activity (7, 70). This suggests that circulating miRNAs (c-miRNAs) may be used as potential biomarkers for diagnosis and treatment of diseases. For example, serum levels of miR-141 have allowed researchers

to distinguish between prostate cancer patients and healthy controls (81), whereas circulating miR-21 has been consistently found to be upregulated in patients with osteoporotic fractures (91, 116). However, the roles of c-miRNAs in osteoporosis, fractures and aging muscles are not fully understood.

To date, the relationship between c-miRNAs and bone status and subsequent risk of fractures remains to be established. Given that current bone turnover markers are mainly derived from the synthesis and degradation of type I collagen from bone matrix, 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. Given 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 (68). Finally, given that bone and muscles are connected in mechanotransduction and metabolic signaling, and both osteoporosis and sarcopenia are key contributors to fractures, it is necessary to include sarcopenia status in fracture risk model. Therefore, the purpose of this study was to identify specific c-miRNAs that are associated with bone and muscle status in postmenopausal women (60-85 years) and to evaluate the use of these c-miRNAs as biomarkers of osteoporosis and sarcopenia.

Research Questions

The specific research questions of this study were as follows.

1. Are there significant differences (upregulation/downregulation) in bone-specific c-miRNAs (miR-21, -23a, -24, -100, and -125b) in postmenopausal women with and without osteoporosis?

2. What are the relationships between these bone-specific miRNAs and bone mass, bone strength?
3. Are there significant differences (upregulation/downregulation) in skeletal muscle-specific c-miRNAs (miR-1, -133, and -206) in postmenopausal women with and without sarcopenia?
4. What are the relationships between these muscle-specific miRNAs and muscle mass, muscle strength, and muscle power?

Hypotheses

1. I hypothesized that bone-specific c-miRNAs would be upregulated in osteoporotic women based on target genes that had been identified or predicted from in vitro or animal studies.
2. I hypothesized that these bone-specific miRNAs would be associated with lower bone mass and lower bone strength.
3. I hypothesized that skeletal muscle-specific c-miRNAs would be upregulated in sarcopenic women based on target genes that had been identified or predicted.
4. I hypothesized that these skeletal muscle-specific miRNAs would be negatively associated with muscle mass, muscular strength and power.

Sub Questions

1. Are bone turnover markers, CTX, TRAP5b and PINP, associated with bone-specific miRNAs?

Sub Hypotheses

1. I hypothesized that bone resorption markers CTX and TRAP5b would be positively associated with miRNAs that inhibit osteoblastogenesis (e.g. miR-23a, miR-100) or stimulate osteoclastogenesis (e.g. miR-21), whereas the bone formation marker PINP would be negatively associated with these miRNAs in postmenopausal women.

Significance of the Study

Research on miRNAs is fairly new, thus it opens up exciting possibilities for novel diagnosis and treatment of diseases. The measurement of c-miRNAs may serve as potential biomarkers of diseases of bone and skeletal muscle as well as risk of fractures. However, c-miRNAs that regulate bone and muscle metabolism are not fully identified and the role of miRNAs in aging is not known in depth. The measurement of serum miRNAs is novel, and it reflects the regulation of gene expression, thus providing the underlying cellular and molecular process involved in bone turnover and muscle aging. Therefore, the significance of this study is to identify c-miRNAs in postmenopausal women with osteoporosis and sarcopenia to help develop new and reliable serum markers of fragility, which would be a great progression in clinical diagnoses of aging-related diseases.

Assumptions

1. Participants honestly and accurately completed all the questionnaires.
2. Questionnaires used in this study were valid and reliable for use in this population.
3. Participants accurately followed the instruction of fasting for at least 8 hours prior to blood draw.

4. miRNAs in the blood samples were not degraded during storage in -80℃ freezer.

Delimitations

1. The findings of this study only apply to community-dwelling postmenopausal women ages 60-85 years.
2. Participants were recruited from Norman and Oklahoma City, Oklahoma areas.
3. Individuals with metal implants at hip or spine were excluded from the study.

Limitations

1. Participants were volunteers and thus may not be truly representative of the population.
2. Measurements only applied to the selected five bone- specific (miR-21, -23a, -24, -100, and -125b) and three muscle-specific (miR-1, -133, and -206) miRNAs.
3. Participants were limited to 350 lbs and 6'4'' due to DXA machine capacity.
4. History of hormone replacement therapy or seasonal variation were not controlled.

Operational Definitions

1. Appendicular Skeletal Muscle Mass (ASM, kg): Fat-free lean mass at arms and legs (9).
2. Areal Bone Mineral Density (aBMD, g/cm^2): The amount of bone mineral per unit of two-dimensional projected area (76).
3. Argonaute (AGO) family proteins: Proteins that associate with small RNAs and function as effectors in RNA silencing (49).
4. Bone Morphogenetic Proteins (BMPs): A group of growth factors that are able to induce endochondral bone formation (76).

5. Bone formation: The building of new bone by osteoblasts (76).
6. Bone Mineral Content (BMC, mg): also called bone mass; the amount of mineral mass measured in a bone, bony area, or the body in grams (76).
7. Bone Strength Index (BSI, mm^3): The density weighed polar section modulus of given bone cross-section. It gives a measure of compressive bone strength at the metaphysis (120).
8. Bone turnover: The amount of bone or the fraction of it that is replaced by new bone (76).
9. Bone turnover markers (BTMs): Enzymes (e.g. acid phosphatase) reflecting bone metabolic activity or bone matrix degradation products released into the circulation during bone resorption (76).
10. Bone resorption: The breakdown of bone tissue by osteoclasts (76).
11. Cortical bone: Dense, compact bone that is 80-90% calcified and fulfills mainly mechanical and protective functions for the body, such as the shafts of the long bones of the arms and legs (76).
12. Complementary DNA (cDNA): A double-stranded DNA synthesized from a mRNA template using reverse transcriptase (12).
13. C-telopeptide of Type I collagen cross-links (CTX): A bone resorption marker that can be measured in the serum to determine the degradation of products of type I collagen (76).
14. Endogenous Control: A molecule that is present in the RNA or DNA sample which is stable across multiple samples (12).
15. Falls: Unintentionally comes to rest on the ground, floor, or other lower level (137).

16. Menopause: Cessation of menstruation, usually occurs at 48-50 years of age in healthy women (111).
17. MicroRNA (miRNA): Short, non-coding, single-stranded RNA molecules made of 20-24 nucleotides that negatively regulate protein expression (74).
18. Osteoblast: Bone cell responsible for bone formation (76).
19. Osteoclast: Bone cell responsible for bone resorption (76).
20. Osteocyte: A mature bone cell, formed when an osteoblast is embedded in bone matrix it has secreted, composing 90-95% of all bone cells (76).
21. Osteopenia: BMD T-score that is less than -1 and greater than -2.5 SD compared to the young adult reference value (88, 94).
22. Osteoporosis: BMD T-score more than 2.5 SD below the young adult reference value. Bone is fragile, and bone fracture risk is increased (88, 94).
23. Peripheral Quantitative Computed Tomography (pQCT): Peripheral QCT, an equipment with a lower cost and lower radiation exposure for measuring cortical and trabecular volumetric BMD (vBMD), bone geometry and SSI, which can give a true description of the cross-sectional geometry and bone composition in contrast to the planar description via DXA (120).
24. Periosteal circumference: An important indicator of bone size and closely related to bone strength. It can be directly determined through pQCT (120).
25. N-terminal propeptide of type I procollagen (PINP): A bone formation marker that is derived from the cleavage of type I procollagen molecules (76).

26. Real-time polymerase chain reaction (RT-q-PCR): A laboratory technique of molecular biology based on PCR, which monitors the amplification of a targeted DNA or cDNA molecule during the PCR in real-time.
27. Receptor activator of nuclear factor kappa-B ligand (RANKL): A type II membrane protein that is known to stimulate osteoclast formation and activity (16)
28. Runt-related transcription factor 2 (RUNX2): A protein that is encoded by the RUNX2 gene in humans and associated with osteoblast differentiation (76).
29. Sarcopenia: Skeletal muscle mass index more than 2 standard deviations below the reference population (9).
30. Skeletal Muscle Mass Index (SMI, kg/m^2): appendicular lean muscle mass (kg) divided by height squared (m^2).
31. Stress Strain Index (SSI, mm^3): A cortical density weighed section modulus of the bone. It gives a measure of bending and torsional strength of diaphyseal sites.
32. T-score: Standard deviation units in relation to the average of healthy Caucasian young females.
33. Threshold Cycle (Ct): In real-time PCR, the number of cycles required for the fluorescent signal to cross the threshold (i.e. background level) (12).
34. Trabecular bone: also called cancellous bone; spongy bone enclosing spaces filled with bone marrow, blood vessels and connective tissue, and is only 10-20% calcified. It fulfills primarily metabolic functions for the body (76).
35. Volumetric Bone Mineral Density (vBMD, g/cm^3): The BMC per cross-sectional area of a bone (99).

36. Wnt signals: a family of signaling proteins that participate in mitogenic stimulation, cell fate specification, and differentiation, including the canonical Wnt pathway and the noncanonical Wnt pathway (76).
37. Z-score: standard deviation units above or below what is normally expected for an individual of the same age, sex, weight, and ethnic or racial origin.

Chapter 2 Review of Literature

Introduction

The world is aging. Aging is accompanied with body composition changes, functional decline, and age-related diseases, such as osteoporosis and sarcopenia. Osteoporosis is a prevalent public health disease without overt symptoms, and patients typically come to clinical attention only after suffering a fracture. Sarcopenia, the age-related loss of muscle mass and function, increases the risk of mortality and decreases quality of life. It is widely supported that fractures are related to low bone mineral density (BMD), while fractures also occur as a result of falls. Therefore, both osteoporosis and sarcopenia contribute to fracture risk. Bone and muscle are closely linked, thus it becomes more evident that in addition to measuring BMD, other non-skeletal risk factors are important to predict risk of fracture and severity of osteoporosis.

MicroRNAs (miRNAs), discovered two decades ago, are short non-coding, single-stranded RNA molecules, 20-24 nucleotides (nt) in length that negatively regulate posttranscriptional protein expression. So far, 2588 (miRBase.org) miRNAs have been identified in humans and 1881 of them have been fully sequenced (46). A single miRNA may target multiple genes, and miRNAs in humans may have a direct influence on at least 30% of genes in the human genome according to computer model. It is well studied and documented that miRNAs are associated with cancer and cardiovascular diseases (81, 100). Some miRNAs have been identified to regulate muscle synthesis and bone osteogenesis, but their roles in osteoporosis and sarcopenia are not established. The purpose of this review is to examine current findings on the prevalence of osteoporosis and sarcopenia in aging, and the role of miRNAs in

osteoporosis and sarcopenia in aging populations. The literature is presented in the following sections: 1. Age-related Bone Loss, 2. Osteoporosis Diagnosis and Intervention, 3. Age-related Changes in Skeletal Muscle, 4. Fracture Prevention, 5. miRNAs in Bone and Muscle.

Age-related Bone Loss

After reaching peak bone mass during 20-30 years in adults, BMD starts to progressively decline, which has been observed in numerous population based studies (32, 51, 52, 105). Riggs et al. (105) measured BMD at radius and lumbar spine in healthy Caucasian women aged 20-88 yr (n=139, mean 52 yr) using single and dual photon absorptiometry, and the measurements were repeated 2-6 times during 0.8-3.4 yrs follow-up. They found that BMD at radius remained stable before menopause but significantly declined by 1.01% per year after menopause. BMD at lumbar spine decreased 1.32% per year before menopause and 0.97% per year after menopause. The Framingham osteoporosis study measured BMD at the femur and radius using Lunar SP2 and DP3 absorptiometry in older participants (n=1102, mean age 76 yr) in the late 1980s. Hannan et al. (51) found that BMD at both sites significantly decreased with aging at similar rates for both sexes, even after adjusting for height and weight. After a 4-year of follow-up, Hannan et al. (52) reported that women lost BMD at 1.2% per year at the radius and 0.86% per year at the femoral trochanter whereas men lost 0.9% per year and 0.04% per year, respectively. The study of osteoporotic fractures by Ensrud et al. (32) measured BMD at total hip using DXA and calcaneus (heel) using single photon absorptiometry in Caucasian women over 65 yr (n=5698). They found that bone loss increased with aging at both sites. At the total hip, the rate of decline in BMD in those

subjects over 85 yr was up to 4-fold greater than those 67-69 yr. Unsurprisingly, hormone replacement therapy users had a slower rate of bone loss compared to nonusers at equivalent ages.

BMD in most studies was measured by DXA. However, DXA measurements are two dimensional, and thus cannot distinguish cortical and trabecular bones, or measure bone microarchitecture or strength. Another technology, central quantitative computed tomography (QCT) or peripheral QCT (pQCT), provides noninvasive measurement to evaluate bone "quality" *in vivo*. Riggs et al. (102) measured trabecular vBMD at lumbar spine by QCT (Light Speed QX-I/Ultra) and trabecular and cortical vBMDs at distal radius and tibia by pQCT (Densiscan 1000) in participants aged 20-97 years (n=553) at baseline and after a 3-year of follow-up. They found that the loss of trabecular vBMD began in young adulthood and was independent of sex steroid levels, whereas cortical bone loss did not begin until middle or even older ages and was associated with sex steroid deficiency. The loss of trabecular bone was greater in the lumbar spine as compared to the peripheral sites, and women generally had greater bone loss than men, particularly during menopause transition. On average, women lost about 1.6% of trabecular vBMD each year at lumbar spine before age 50 and 2.6% after age 50, whereas men lost about 0.84% and 1.85% before and after age 50, respectively. This means that, accumulatively, women lost 37% of trabecular bone and 6% of cortical bone before age 50 compared to 42% and 15% of each after age 50. Another study by Riggs et al. (104) estimated that women could lose as much as 55% of trabecular vBMD over their lifespan, which was significantly higher than the loss of 46% in men, especially considering that women had a lower bone mass than men.

Several mechanisms are involved in age-related bone loss, including oxidative stress, sex steroids deficiency, and apoptosis. (56). Intracellular metabolism generates reactive oxygen species (ROS), such as superoxide anions (O_2^-), hydroxyl radicals (HO^-), and hydrogen peroxide (H_2O_2). As aging, the antioxidant system is impaired, such as the ROS-scavenging enzyme superoxide dismutase (SOD), resulting in accumulation of ROS and further increasing oxidative stress. In a mouse model of SOD deficiency, the SOD knockout mice have a higher level of oxidative stress, reduced BMD and bone strength, and a higher rate of apoptosis, resulting in fewer osteoblasts than in wild-type mice (87, 122). In addition, the accumulation of ROS causes retention of the forkhead box O (FoxO) family of transcription factors in the nucleus and elevated $p66^{Shc}$ signaling activity, both of which lead to reduced bone formation and age-related bone loss (4, 86).

Sex steroids play a critical role in regulating bone turnover rate. During menopausal transition, serum estradiol levels decrease by 85-90% (66), bone resorption increases by 90%, whereas bone formation only increases by 45% (40). The net imbalance in bone formation and resorption results in rapid bone loss, particularly in trabecular bone (40, 103). Estrogen stimulates differentiation of bone marrow stromal cells to the osteoblast lineage as well as differentiation of preosteoblasts to osteoblasts. In addition, estrogen inhibits osteoblast and osteocyte apoptosis but stimulates the osteoblastic production of growth factors, such as insulin like factor-1(IGF-1), transforming growth factor-β (TGF-β), as well as procollagen synthesis (33, 98). Age-related estrogen deficiency is linked to a significant increase in stromal cell differentiation into fat lineage and greater marrow adiposity, which leads to fewer

stromal cells differentiating into osteoblast lineage (83). More recently, it has been discovered that estrogen inhibits serum levels of sclerostin, a potent inhibitor of the Wnt signaling pathway, thus maintaining bone formation (80, 82). Estrogen also regulates osteoclast development effectively by inhibiting production of the receptor activator of nuclear factor kappa-B ligand (RANKL) by bone marrow stromal or osteoblast precursor cells (T & B cells), and stimulating production of osteoprotegerin (OPG, RANKL decoy receptor) by osteoblast lineage cells to limit exposure of osteoclast lineage cells to RANKL (65). In postmenopausal women, the dramatic decline in estrogen levels alters the relative RANKL/OPG ratio, resulting in elevated osteoclast development and activity.

Bone loss is more pronounced in trabecular than cortical bone for two reasons: 1) trabecular bone loss begins in young adulthood, and 2) trabecular components have a greater surface area where bone resorption occurs (103). Women have rapid trabecular bone loss as well as overall bone loss during the menopausal transition compared to men at corresponding ages (67). The significant changes in trabecular microarchitecture are related to decline in bone strength and ultimately increase fracture risks in postmenopausal women. This leads to fractures commonly occurring in trabecular-rich regions, such as distal forearms and vertebrae, particularly in females during early menopause.

Osteoporosis Diagnosis and Intervention

Although the term “osteoporosis” was originally used by French pathologist and surgeon Jean Lobstein in 1835, the first international consensus of a conceptual definition of osteoporosis was achieved in 1993, stating that “osteoporosis is a systemic

skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture" (94). In 1994, the World Health Organization (WHO) proposed using BMD as the diagnostic criterion for osteoporosis. BMD values more than 2.5 standard deviations (SD) below the mean of a young reference population (T-score $\leq$ -2.5) were deemed to indicate a 20% risk of a major fracture within 10 years (88). Later, guidelines were updated using femoral neck measurements instead of multiple sites for BMD (60). The Third National Health and Nutritional Examination Survey (NHANES III) database for femoral neck measurements in Caucasian women aged 20-29 years were used as the reference population. Over the past two decades, diagnosis and assessment of osteoporosis have been updated several times (62, 75). In 2001, the National Institutes of Health Consensus Development Panel on Osteoporosis updated the definition of osteoporosis, defining it as "a skeletal disorder characterized by compromised bone strength predisposing a person to an increased risk of fracture", and this definition is still valid and useful to this day (97).

Conventionally, a BMD T-score $\leq$ -2.5 at the lumbar spine or hip is used as the intervention threshold of osteoporosis. It is reported that age-adjusted risk of hip fracture increased 2.6 fold with every SD decrease in hip BMD (77). However, this fracture risk model disassociates bone strength from diagnosis, and a significant proportion of fragility fractures occur in individuals with a BMD T-score above -2.5. In fact, approximately 50% of hip fractures occur in individuals without osteoporosis (133). In addition, a given T-score of -2.5 has different impact on fracture probabilities throughout the lifespan. For example, the 10-year probability of fracture is consistently

higher in women with osteoporosis (T-score ≤ -2.5) than those without osteoporosis (T-score > -2.5). However, the difference between categories decreases after age 65 and individuals with osteoporosis have an even lower rate of fracture than those without osteoporosis after age 78 (61). Furthermore, fracture rates vary from country to country. For example, at a given 10-year probability of major fracture of 20%, the BMD T-score is -2.5 in the US, whereas it is approximately -2.0 in Iceland and -4.5 in Venezuela (75). Therefore, the use of BMD is not completely appropriate to all populations as an intervention threshold. A better intervention threshold is needed for diagnosis and treatment to reduce healthcare costs and improve quality of life for those suffering from this disease.

Age-related Changes in Skeletal Muscle

Aging is accompanied with declines in muscle mass and strength, and this phenomenon has captured researchers' attention in the recent 30 years. In 1989, Irwin Rosenberg pointed out the importance of age-related decline in lean body mass and coined the term "sarcopenia" to describe it (112). Sarco-, from Greek, means "flesh", and -penia indicates "loss". Thus, sarcopenia means loss of skeletal muscle mass. Recently, studies have identified the disconnection between loss of muscle mass and muscle strength in the conventional definition of sarcopenia, thus incorporating muscle strength and physical performance into sarcopenia criteria (23, 27, 37, 45, 125). Since 2009, several consensus operational criteria have been proposed to define sarcopenia, including: the International Working Group (IWG) (2009), the European Working Group on Sarcopenia in Older People (EWGSOP) (2010), the Foundation for the National Institutes of Health (FNIH) (2014), and Asian Working Group for Sarcopenia

(AWGS) (2014). According to the EWGSOP, sarcopenia is a syndrome characterized by progressive and generalized loss of skeletal muscle mass and strength with a risk of adverse outcomes such as physical disability, poor quality of life and death (23).

Several mechanisms are involved in the development of sarcopenia. At the cellular level, there is substantial loss of both muscle fiber size (cross-sectional area) and number, especially the shrinkage of type II fibers. Muscle twitches become smaller and slower due to impaired calcium release (35). Meanwhile, connective tissue content increases within and between fibers, which is also called fat infiltration of muscle. At the metabolic level, key factors of regulating protein balance, such as IGF-1 and mTOR kinase, are decreased (95). At the vascular level, capillary density is decreased due to increased oxidative stress and mitochondrial dysfunction. Inflammatory factors, such as tumor necrosis factor alpha (TNF-α), interleukin 9 (IL-6), and C-reactive protein (CRP), are increased (71, 95). All these factors contribute to the development of sarcopenia. Exercise intervention and protein supplementation have the potential to prevent or delay the development of sarcopenia by increasing growth to reach peak muscle mass and strength and simultaneously slowing down age-related decline (28, 95). Although estrogen plays a critical role in osteoblast/osteoclast biology, the effects of estrogen on skeletal metabolism at the cellular and molecular level remains unresolved.

Due to two operational definitions of sarcopenia, two approaches have been used to diagnose sarcopenia in research. Initially, sarcopenia is classified based on muscle mass alone using the skeletal muscle mass index (SMI), which equals to appendicular skeletal muscle mass (ASM)/ height^2 (m^2). Sarcopenia is defined as an SMI that is 2 SD below the average of the young reference population (9). Based on this

conventional definition, the prevalence of sarcopenia ranges 13-24% among individuals under 70 years and >50% among those over 80 years when compared to a young reference group (9). More recently, the EWGSOP and FNIH suggest combining low muscle mass with either low muscle strength or poor physical performance to diagnose sarcopenia, whereas the IWG recommends combining low muscle mass with low muscle function (23, 37).

Patel et al. (92) measured body composition and physical performance in 103 community-dwelling UK men with an average age of 73 yr. They found that the prevalence of sarcopenia in older people in the UK was 6.8% in men using the EWGSOP definition. Patil et al. (93) studied 70-80 year old community-dwelling women in Finland (n=409), and found the prevalence of sarcopenia was 0.9% using the EWGSOP definition compared to 2.7% using the IWG definition The International Sarcopenia Initiative (2014) reviewed global studies using the EWGSOP definition of sarcopenia and reported that the prevalence of sarcopenia ranged from 1 to 29% in community-dwelling elderly, approximately 10% in the acute hospital-care population, and increased to 14-33% in the long-term care population (24). Generally, the prevalence of sarcopenia is higher in women than men.

The loss of muscle strength is much faster than the loss of muscle mass in the process of aging (45). Grip strength has been widely accepted as the most practical method of measuring muscle strength in a clinical setting. It is an easy and inexpensive screening tool and has been found to correlate with incident disability for activity of daily life (ADL) (2, 42). The cutoffs from EWGSOP for grip strength are 30 kg for men

and 20 kg for women, whereas 26 kg for men and 16 kg for women are used to define weakness in FNIH's definition and in AWGS (21, 23, 27).

Gait speed is the most common measure of lower extremity performance in clinical setting and for epidemiological sarcopenia studies. Since it is reliable, valid, and correlates with physical performance, it is known as an "almost perfect measure" and "the sixth vital sign" (38). Gait speed is a strong predictor of health status and adverse outcomes, and can be used as a single tool to replace the complete set of the Short Physical Performance Battery (SPPB) (48, 128). It can be quickly and accurately assessed by measuring the time taken to walk a given distance, such as 4 m, at a normal pace. The EWGSOP utilizes 0.8 m/s to define functional deficiency, whereas the IWG uses 1.0 m/s as the cutoff.

Recently, more studies have begun to measure muscle power to examine functional changes as a new component of sarcopenia (18). Rittweger et al. (106) assessed jump power through jumping mechanography from participants aged 24-88 years and found that jump power had good test-retest reliability, and was particularly useful in middle-age to older participants. Buehring et al. (18) found that jumping power correlated with grip strength, with an even stronger correlation with age in elderly participants over 70 years of age, thus concluding that jump power could potentially detect changes in muscle function and better monitor sarcopenia. Singh et al. (121) found community-dwelling individuals, aged 55-75 years, classified as sarcopenia using the conventional definition had significantly lower jump power but not muscle strength compared to non-sarcopenia individuals. Bean et al. (2003) analyzed the population based cohort InCHIANTI study (n=1032, mean 74.2 yr), and found poor

muscle power in legs measured by knee extension was more associated with poor mobility (gait speed < 0.8 m/s) than leg strength.

Currently, one of the most challenging issues is the lack of consensus about the clinical definition of sarcopenia and assessment of muscle function and muscle strength among consensus groups and studies. Due to different criteria used for diagnosis, the prevalence of sarcopenia varies greatly, which may lead to different conclusions and implications for treatment. Bischoff-Ferrari et al. (13) compared the current seven available definitions of sarcopenia using the original Boston cohort, which included 445 community-dwelling older adults over 65 years of age with a 3 year follow-up. They reported that the prevalence of sarcopenia decreased from 11% to 7% when using the EWGSOP definition instead of the conventional one. The elderly with sarcopenia based on the conventional and the EWGSOP definitions had the highest possibility of falls compared to the non-sarcopenia ones, with odds ratio of 1.54 and 1.82, respectively, thus suggesting that these two definitions were the best to recognize the risk of falls among sarcopenic elderly.

Fracture Prevention

Fracture is the primary clinical end point for osteoporosis treatment. The risk of fracture increases remarkably with age, with more than two thirds of fractures occurring in women over 65 years old. Vertebral and hip fractures are the major osteoporotic fractures that have high morbidity and mortality and societal burden, and they are most common in postmenopausal women due to pronounced decline of estrogen. Vertebral fractures are common, with a prevalence of 25-50% in women over 50 years of age and particularly high over 75 years old, leading to back pain and reduced physical function,

but only 30% of them are clinically recognized. Hip fractures are associated with 8.4% to 36% mortality in the first year after fracture (1).

It is well established that the risk of fractures is about four-fold higher in osteoporotic patients compared to individuals with normal BMD. However, fractures are not only associated with bone loss, or the degree of trauma. In the US cohort Study of Osteoporotic Fractures (124), BMD of community-dwelling women aged 65 and older (n=9704) were measured at baseline, and participants were contacted every 4 months via phone or mail to record all types of fractures with at least 8 years of follow-ups. The researchers found that only 15% of all types of fractures were attributable to osteoporosis, although women with low BMD have higher prevalence of all types of fractures. Osteoporosis at spine or hip contributed to 8% to 44% of fractures, and the relationship between BMD and fractures in women over 65 years old was moderate (124). Another epidemiology study in England and Wales investigated the age and gender specific prevalence of fractures in a sample of general practice research database. It was found that the relative risk of hip fracture in elderly people aged 60-80 years increased 13 fold whereas only two fold increased risk was due to aging (130). Therefore, it is apparent that not only bone loss but also other factors contribute to the increased fracture risk with advancing age. Vertebral fractures are associated with decreased BMD and deteriorated bone microarchitecture that are not related to menopause, and they are a stronger fracture predictor than low BMD or other fractures (14). Besides osteoporosis, additional interventions are needed to find effective prevention strategies for fractures, such as fall prevention and other fracture risk factors.

A major non-skeletal factor that contributes to fracture risk is falls. Falls are defined as "unintentionally coming to rest on the ground, floor, or other lower level" (137). Although most falls do not result in fractures, over 90% of all fractures occur after a fall. Therefore, fall prevention is the key to prevent fractures. Hip fractures tend to occur in less active people, whereas forearm and humerus fractures are more common among people who are more active. Patients with osteoarthritis, particularly at weight-bearing joints, have a higher risk of fractures due to falls, although they have higher bone density compared to those without osteoarthritis. Risk factors of falls are: gait instability, weakness, visual/cognitive impairment, home hazards and circumstances (e.g. snow, ice), and side effects from drugs (e.g. antidepressants, anticonvulsants) (111).

Gait instability, the major intrinsic risk factor of falls, is often due to muscle weakness, or sarcopenia. Weaker muscle decreases muscle strength and power, impairs physical performance, leading to increases in falls and fractures. Much the same as osteoporosis predicting risk of bone fractures, sarcopenia is associated with increased risk of falls, which consequently increases fractures, disability, and mortality (108). Therefore, sarcopenia should be the next focus in fracture prevention.

Bone and skeletal muscle are two fundamental systems in the human body that interact with each other. Mechanically, muscle attaches to bone and generates mechanical loading on bone during muscle contraction. This mechanotransduction seems to regulate both skeletal muscle and bone metabolism (44). Moreover, there is accumulating evidence that bone and muscle are regulated by several signaling pathways that affect both in growth, diseases and aging. For instance, BMP signaling

not only regulates osteoblasts and osteoclasts to maintain bone homeostasis, but also plays a role in myogenesis and skeletal muscle mass (114, 115). Vitamin D is also a key regulator in bone remodeling process and calcium and phosphate homeostasis. Recently, research revealed that vitamin D receptor is also expressed in skeletal muscle and low vitamin D levels are associated with increased risk of falls in the elderly (47). IGF-1, which is important in muscle hypertrophy, also was found to stimulate osteoblast differentiation and bone formation (10). Similarly, mTORC1, a major regulator of protein synthesis, might also be a potential regulator in bone (44). Osteocalcin, exclusively secreted by osteoblasts to regulate bone mineralization and maintain calcium homeostasis, also has receptors in the skeletal muscle indicating that it might have an effect on muscle strength as well. It is well known that Wnt signaling plays an important role in bone development and homeostasis, however, recent research also found it might be related to myogenesis in skeletal muscle (113). Overall, bone and muscle are two connected tissues with mechanical and metabolic interactions. Therefore, it is important to recognize the connection between osteoporosis and sarcopenia and the role of sarcopenia in fracture prevention.

Numerous studies have shown that exercise improves BMD, muscle strength and functional performance in community-dwelling older adults, which further reduces falls as well as the risk of fractures (63, 85, 119, 138). Even unsupervised home exercise programs reduce falls, however, it is better to have an exercise program that involves strength and balance training. In a study by Nelson et al. (85), the first resistance training study on bone density two decades ago, postmenopausal women aged 50-70 were randomly assigned to a one year high intensity resistance training

group (n=20, exercise twice per week, 5 exercises) or control group (n=20). The researchers found that the resistance training group significantly increased their femoral neck and lumbar spine BMD after a year of training, whereas the control group had significant bone loss after one year. Meanwhile, muscle strength and balance were improved in the resistance training group but decreased in the controls, compared to their baseline. Zhao et al. (138) conducted a meta-analysis to investigate the effects of resistance exercise (≥ 6 months) on BMD in postmenopausal women, and effect size was estimated using the calculation of standardized mean difference. They found that overall resistance training (24 studies, 1769 postmenopausal women) significantly improved femoral neck and lumbar spine BMDs with effect sizes of 0.303 and 0.311, respectively. However, when comparing the resistance training alone intervention and combined resistance and weight-bearing exercise intervention, only the combined resistance training intervention significantly increased femoral neck and lumbar spine BMD, with effect sizes of 0.411 and 0.431, respectively. In a meta-analysis by Kelly et al. (63), they examined the effects of ground and joint impact exercise intervention (≥24 wk) on BMD in postmenopausal women using the standardized mean difference as effect size as well. They reported that ground/joint impact exercise had small but significantly beneficial effects on femoral neck and lumbar spine BMDs, with effect sizes of 0.288 and 0.179, respectively, which could slightly reduce the risk of osteoporotic fracture.

Kemmler et al. (64) conducted a meta-analysis on the effects of exercise (resistance, endurance, balance, etc.) intervention on fracture prevention in the elderly. The relative risk (RR) of fractures in each study was calculated. However, the exercise

intervention groups were not found to have differences in overall fracture (RR=0.49, p=0.28) and vertebral fracture (RR=0.56, p=0.26) compared to the older controls. In another meta-analysis by Polidoulis et al. (96), they reviewed six exercise intervention studies (5-12 months) in postmenopausal women that measured bone strength by pQCT. They reported that lower body exercise significantly improved trabecular vBMD at distal tibia and cortical vBMD at tibia shaft, suggesting that exercise may diminish bone loss in postmenopausal women.

Cruz-Jentoft et al. (24) reviewed seven studies that examined the effects of exercise intervention on skeletal muscle in older adults. They found that 3-18 months of resistance training alone significantly increased muscle mass, strength and functional performance, such as gait speed and chair rise. They also reported that overall exercise (endurance, resistance, balance) generally improved muscle strength and functional performance in community-dwelling older adults.

MiRNAs in Bone and Muscle

In 1993, Dr. Victor Ambros discovered the first miRNA, lin-4, which is responsible for normal larval development of *C. elegans* (72). Soon after, Dr. Gary Ruvkun's lab found that lin-4 RNA is complementary to 7 sites in the lin-14 3'UTR, thus downregulating the expression of the lin-14 protein (136). In 2000, Dr. Gary Ruvkun discovered a second miRNA, let-7, which is conserved across animals, including humans, that started the small RNA revolution (101). Since then, the research of miRNAs is emerging, and miRNAs are recognized as a distinct class of biological regulators in normal gene expression as well as disease.

MiRNA genes are transcribed by RNA polymerase II to generate a long, hairpin shape termed primary miRNA (pri-miRNA). Then the nuclear ribonuclease III enzyme in the nucleus, Drosha, processes the pri-miRNA into a 60-70 nucleotide stem-loop structure called precursor miRNA (pre-miRNA). Exported into the cytoplasm by exportin-5, the pre-miRNA is processed into an unstable double-stranded miRNA by a second ribonuclease III enzyme called Dicer. The miRNA duplex is then incorporated into the RNA-induced silencing complex (RISC), which includes an argonaute protein. One strand of the pre-miRNA is degraded, while the other strand becomes mature miRNA which binds to a complementary site in the 3' untranslated region (UTR) of the target mRNA to destabilize the mRNA or repress translation (74).

A tissue-specific miRNA is defined as "a mature miRNA that is expressed at least 20-fold higher in a specific tissue than the average expression of all other tissues" (78). Sempere et al. (117) first identified 3 muscle-specific miRNAs in mice and humans: miR-1, -133, and -206, which regulate cardiac and skeletal muscle development, and proliferation and differentiation of myoblast. MiR-1 and -133a are well established to be highly expressed in cardiac and skeletal muscle, whereas miR-206 is expressed in skeletal muscle only, primarily in slow twitch fibers (69). Additional muscle-specific miRNAs recently identified are: miR-208a, -208b, -486 and -499. In particular, miR-208a is specifically found in cardiac muscle. It is reported that miR-1 is related to inhibition of myoblast proliferation, which counteracts the promotion of proliferation by miR-133a (3).

McCarthy et al. (79) applied mechanical overload to the plantaris muscle of mice for 7 days to induce muscle hypertrophy and analyzed several miRNAs from

plantaris muscle tissue. They found that after 7 days of overload, the expression of Drosha and Exportin-5 increased by 50%, and the expression of pri-miRNA-1-2, -133a-2 increased by 2-fold. Although the expression of miR-206 was unchanged, the expression of miR-1 and -133a decreased by 50%, which suggested that the overload may stimulate the expression of genes known to be related to muscle growth. Elia et al. (31) reported that miR-1 targeted IGF-1 pathways in mice model to regulate skeletal muscle hypertrophy, as it was partially complimentary to 3' UTR. Allen et al. (3) investigated muscle-specific miRNAs in mice after 12 days of spaceflight. They found that miR-206 in gastrocnemius muscle decreased significantly by 50% compared to preflight expression levels. Although miR-1 or -133a were not changed, the ratio of miR-1 and -133a increased significantly after spaceflight, suggesting potential inhibition of cell proliferation.

Most of the findings mentioned above were based on the analysis of muscle tissue in animals and humans. More recently, researchers began to measure c-miRNAs in humans as markers of physical fitness, stress responses, risk and status of diseases, although results regarding the effects of exercise on muscle-specific c-miRNAs are not consistent so far (5). In a study by Baggish et al. (6), 10 participants underwent 90 days of rowing training to improve their aerobic capacity. Prior to and post 90 days training, peak oxygen consumption (VO_{2max}) was tested via cycle ergometer, and eight miRNAs involved in angiogenesis, inflammation, cardiac and skeletal muscle function, and hypoxia responses were measured in serum: miR-20, -21, -133a, -146a, -210, -221/-222, -328. Compared to resting baseline levels, they found that circulating miR-146a, -222, -21, and -221 were significantly increased after acute cycling at baseline as well as at

rest after 90 days of aerobic training. However, only circulating miR-146a and -222 after acute cycling were increased post-training, whereas miR-21 and -221 were not changed, suggesting a possible maximal ceiling expression of these specific c-miRNAs. In addition, they found that miR-146a was significantly positively correlated with VO_{2max} (r=0.63), suggesting that serum miR-146a may serve as a quantitative biomarker of cardiorespiratory fitness. Banzet et al. (8) measured muscle-specific miRNAs (miR-1, -133a/b, -208a/b, and 499) and muscle related miRNAs (miR-181 and 214) in nine recreationally active and healthy young men in response to a single bout of 30 min uphill or downhill walking on treadmill (1 m/s, 25% of grade, 12% of body weight loaded backpack). They reported that the levels of circulating miR-1, -133a, -133b and -208b were significantly elevated 6 hours post downhill walking compared to baseline, and no significant changes were overserved in uphill walking. This finding suggested that responses of miRNAs were related to exercise mode, with no changes in concentric exercises but significant decreases in eccentric exercise. Gomes et al. (43) measured c-miRNA levels before and after half-marathon, and they reported that circulating miR-1, -133a, and -206 were significantly upregulated after a half-marathon run.

MiRNAs also regulate osteogenesis and are associated with bone diseases such as osteoporosis, osteoarthrosis, and rheumatoid arthritis. Bone-regulating miRNAs are those expressed in osteoblast lineage cells for regulation of bone formation by either direct repression of inhibitors of osteoblast differentiation or by their response to osteogenic signals, such as BMP, to promote osteogenesis. More and more miRNAs have been identified to regulate osteoblastogenesis and bone formation by targeting inhibitors of osteogenesis or osteogenic factors (74). Some miRNAs regulate

osteoblastogenesis, such as miR-20a (stimulates BMP/RUNX2 signaling pathway), miR-29a (stimulates Wnt signaling pathway), miR-23a (inhibits RUNX2 signaling pathway), and miR-100 (inhibits BMPR2 signaling pathway), whereas some miRNAs regulate osteoclastogenesis, such as miR-21. MiR-21 and miR-125b are also involved in the differentiation of osteocytes from human mesenchymal stromal cells (hMSC) (84).

In osteoporosis, miRNAs regulate the differentiation, proliferation and apoptosis of bone cells (84). Seeliger et al. (116) measured miRNAs in serum and bone tissues from patients aged over 50 yr with hip fractures compared to nonosteoporotic controls. They analyzed two miRNA arrays that contained miRNAs from osteoporotic patients and controls, respectively, and found 9 c-miRNAs (miR-21, -23a, -24, -93, -100, -122a, -124a, -125b, and -148a) that were significantly upregulated in osteoporotic fracture patients compared to controls. Additionally, 6 miRNAs (miR-21, -23a, -24, -25, -100, and -125b) in bone tissue were found significantly upregulated in osteoporotic fracture patients. Overall, 5 miRNAs were found significantly upregulated in osteoporotic patients in both serum and bone tissue compared to controls, including miR-21, -23a, -24, -100, and -125b. Panach et al. (91) measured c-miRNAs in patients with hip fractures and controls with severe osteoarthritis at the hip. They found 3 c-miRNAs, including miR-122, -125b, and -21, that were significantly upregulated in fracture patients compared to osteoarthritic controls. In particular, the level of miR-21 was correlated with the level of CTX ($r=0.76$, $p<0.000001$), a bone resorption marker.

Aging affects miRNA expression. Drummond et al. (29) compared miRNA expression in skeletal muscle following acute resistance exercise and essential amino acids ingestion in young (mean 29 yr) and older (mean 70 yr) men. They found that

primary miRNAs of pri-miR-1-1 and -1-2, pri-miR-133a-1 and -133a-2 were significantly higher in muscle tissue in the elderly compared to young men at baseline. There were no differences in miRNA biogenesis pathways, including Drosha, Exportin-5, Dicer mRNA, or mature miRNA found between young and old men at baseline. MiR-1 was reduced in young but not older men following exercise stimulus, indicating that aging results in a dysregulated miRNA response after anabolic stimulus. More recently, Drummond et al. (30) conducted a muscle tissue microarray study in young and older men and confirmed that let-7b and -7e were significantly higher in the older group than the young men. The let-7 family was predicted to be associated with cell cycle control and was validated that there was downregulation of regulators of cell cycle proliferation in the older group. Overall, there was lower expression of genes related to cell cycle regulation in skeletal muscle in older men, which may have been regulated by the let-7 family. This study could have been more interesting if they not only examined muscle tissue, but also measured serum miRNAs and compared them in the young and older groups.

So far, no study has compared bone-related miRNAs in serum between younger and older populations. Rivas et al. (107) compared the acute miRNA responses to resistance exercise between younger and older men. Participants completed 3 sets of 10 repetitions of knee extension and leg press exercises at 80% 1-RM and muscle biopsy was obtained 6 hours post exercise. They found that expression of 21 miRNAs were altered by exercise in the younger men whereas there were no changes in the older men. In addition, expression of 175 protein-coding genes was altered by exercise in the

younger men as compared to only 42 genes in the older men. MiR-126 appeared as an important regulator in muscle growth and IGF-1 pathways.

MiRNAs are being recognized as important regulatory molecules in a large number of biological process. Studying miRNAs is a new direction in the research of osteoporosis and sarcopenia, and understanding miRNA expression profiles and dynamic regulation is able to provide new mechanisms in basic research, potential novel diagnostic biomarkers, and drug targets for treatment of osteoporosis and sarcopenia. However, numerous questions need to be addressed before utilizing miRNAs as a biomarker in osteoporosis and sarcopenia.

Summary

It is important to characterize the molecular mechanisms associated with osteoporosis and sarcopenia. By identifying new signaling pathways, it is likely that miRNAs may inspire novel approaches for the detection of osteoporosis and sarcopenia and provide better therapy so that more options are available for the treatment of osteoporosis and fracture prevention.

Chapter 3 Methodology

This cross-sectional study measured the expression levels of selected c-miRNAs in relation to osteoporosis and sarcopenia statuses in postmenopausal women. In addition, this study examined the relationships between bone-specific miRNAs and bone density, bone strength and bone turnover markers, and muscle-skeletal miRNAs and muscle mass and muscular strength. Finally, this study compared functional performance, muscle mass and strength, and bone density and strength among normal, osteopenia, and sarco-osteopenia postmenopausal women.

Participants

Seventy-five community-dwelling, postmenopausal women aged between 60 to 85 years old were recruited in this study. Fifteen of the participants were measured twice on bone measurements and functional performance tests. Prior to participation, each participant was asked to sign a written informed consent and HIPAA forms. The study was approved by the University of Oklahoma Health Sciences Center (OUHSC) Institutional Review Board (IRB#6971). Participants were recruited from Norman and Oklahoma City areas by mass email, flyers, newspaper advertisements, and by word of mouth. Participants were screened for inclusion and exclusion criteria prior to consenting and testing.

Inclusion Criteria

1. Postmenopausal women, aged 60-85 yr;
2. Community-dwelling individuals.

Exclusion Criteria

1. Current smokers;

2. Individuals with diabetes or uncontrolled hypertension;
3. Individuals taking medications known to affect bone metabolism, such as antidepressants, glucocorticoids;
4. Body weight over 350 lbs or height over 6’4’’;
5. Individuals with recent fractures within 12 months;
6. Individuals with metal implants or joint replacement at hip or spine.

Research Design

This cross-sectional study compared relative expression levels of bone- and muscle-specific c-miRNAs based on statuses of osteoporosis and sarcopenia. Three visits were required in this study: consenting, blood pressure, and questionnaires (visit one, ~1 hr), blood draw and measurement of DXA, pQCT, and familiarization (visit two, ~2.5 hrs), and functional performance tests (handgrip strength, gait speed, jump test, balance) (visit three, ~1 hr). However, to assess reliability and consistency of our measures, a subset of 15 participants (the first 15 participants aged 70-85 yrs who were willing to return for a fourth visit) repeated the measurement of DXA, pQCT, and functional performance in an additional visit (visit four, ~1.5 hr). The fourth visit was 3-7 days after the third visit. The first visit was conducted at Bone Density Laboratory at the University of Oklahoma (OU). During the second visit, the blood draw was performed at OU Goddard Student Health Center, and the rest of the second, third, and fourth visits were conducted in the Bone Density Laboratory at OU. A medical clearance form signed by the participant’s physician was obtained prior to scheduling the second, third and fourth visits.

The independent variables were status of osteoporosis (normal, osteopenia, osteoporosis) and sarcopenia (normal, sarcopenia). The dependent variables for blood analysis were relative expression levels of bone- and muscle-specific c-miRNAs (miR-1, -21, -23a, -24, -100, -125b, -133a, -206), and levels of bone turnover markers. The dependent variables for the DXA were aBMD of total body, lumbar spine, and dual femur, and body composition. The dependent variables for the pQCT were total vBMD, total vBMC, and total area at tibia 4%, 38%, and 66% sites; trabecular vBMD, trabecular vBMC, trabecular area and BSI at tibia 4% site; cortical vBMD, cortical vBMC, cortical area, Ipolar, and SSI at tibia 38% and 66% sites, and muscle cross sectional area (CSA) at tibia 66% site. The dependent variables for functional performance were grip strength, gait speed, and jump power.

Questionnaires

Several questionnaires were filled out by the participants to gather information regarding potential confounding variables that affect bone health and muscle performance, including menstrual history, calcium intake, and physical activity levels. The following questionnaires were used in this study.

1. Health Status Questionnaire – to identify whether the participant meet the inclusion and exclusion criteria for the study and to record medications taken by the participant.
2. Menstrual History Questionnaire – to provide information about menstrual cycle and hormone replacement therapy history.
3. Bone-Specific Physical Activity Questionnaire (BPAQ) – to quantify exposure to bone loading physical activity throughout the lifespan (135).

4. Calcium Intake Questionnaire – to estimate daily calcium intake from diet and supplements.

Body Weight and Height

Height and weight were measured to the nearest 0.5 cm and 0.1 kg, respectively, using a wall stadiometer (PAT #290237, Novel Products, Rockton, IL) and digital electric scale (BWB-800, Tanita Corporation of America, Inc., Arlington Heights, IL).

Blood Pressure

Resting blood pressure was measured by an automatic blood pressure monitor (Omron, Japan) on the left arm. The participant sat in a chair with her back supported, legs uncrossed and feet on the floor. The measured arm was supported with the upper arm at heart level. The monitor was turned on with appropriated cuff size selected. The cuff was placed over the brachial artery and the "START" button was pressed to automatically increase the cuff pressure and start the measurement. The systolic and diastolic blood pressures, and heart rate were recorded. One minute later, the second measurement was performed. If there is more than 5 mmHg difference, a third measurement were performed and the average of the closest two values was used. Hypertension was classified as systolic blood pressure above 140 mmHg or diastolic blood pressure above 90 mmHg. Therefore, participants with systolic blood pressure above 140 mmHg or diastolic blood pressure above 90 mmHg were excluded considering the risk when doing functional performance tests.

Dual Energy X-ray Absorptiometry (DXA)

Lunar Prodigy DXA (GE Healthcare, Madison, MI) was used to measure areal bone mineral density (aBMD) via a series of four bone scans, including the total body,

AP lumbar spine (L1-L4), and dual proximal femur (femoral neck, trochanter, and total hip). 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. Scans were analyzed using the enCORE software, version 16 (GE Healthcare, Madison, WI).

This research study involved radiation exposure from four DXA scans, which ranged from 0.08 to 0.18 mrem for each scan. Thus, the participants were exposed to a total dose of less than 6 mrem of radiation, which is similar to one transcontinental flight across the United States (4-6 mrem) and much less than the typical radiation exposure with conventional chest X-ray and CT scans (25 to 270 mrem) (57). A Quality Assurance (QA) test was performed to calibrate the DXA and ensure that the DXA was working properly at the beginning of each testing day prior to data collection. A standard calibration block was positioned on the DXA table, and then the software ran the remaining QA test automatically until completion.

Prior to DXA scans, the participant was asked to take off shoes, wear minimal clothing and remove all metal, and then lie in the supine position on the table, with the head approximately 2-3 cm below the horizontal line at the top of the table. Hips and shoulders were evenly spaced in the middle of the table, with arms close to the body, and knees and feet secured with one strap each to keep the legs straight. Upon the completion of the total body scan, a foam block was placed under her legs with knees bent at 45-60 degrees. The participant kept hips and upper body straight, pointed out her navel so that the scan arm could be adjusted to 2 finger widths below the navel, and then held her arms upright and the lumbar spine was scanned. Once the scan was completed, the block was removed and the feet were placed onto each side of the foot

brace using the straps. The left leg was positioned straight so that the left hip was scanned first and then the same procedure was conducted with the right leg.

Precision of bone density and body composition in postmenopausal women was assessed by the technician who measured all the participants by DXA (Table 1). The root mean square (RMS) coefficient of variation (CV%) was calculated using the International Society for the Clinical Densitometry (ISCD) Bone Densitometry Precision Calculating Tool. The CV% for lumbar spine, femoral neck, and total hip were within the minimum acceptable precision according to the 2015 ISCD Adult Official Position Stands (118).

Table 1. Precision for Bone Density and Body Composition Measurements by DXA

Variable	CV%
Total Body aBMD	1.27%
Lumbar Spine L1-L4 aBMD	1.80%
Left Femoral Neck aBMD	1.79%
Right Femoral Neck aBMD	1.33%
Left Total Hip aBMD	1.19%
Right Total Hip aBMD	1.00%
BFLBM	1.21%
Fat Mass	1.74%
Body Fat%	1.56%
Arms BFLBM	3.97%
Legs BFLBM	2.29%
ASM	2.08%

ASM: Appendicular Skeletal Muscle Mass

Peripheral Quantitative Computed Tomography (pQCT)

Peripheral Quantitative Computed Tomography (pQCT) is an effective supplement to DXA providing detailed information about the cross-sectional geometry of skeletal sites. It measures the true volumetric values of BMD and discriminates between the trabecular and cortical components of bone. Also, pQCT scans assess bone stress strain index (SSI) at multiple sites, thus providing additional information about bone density, bone composition, and bone strength.

A XCT-3000 bone scanner (Stratec Medizintechnik GmbH, Pforzheim, Germany) was used for the epiphyseal and diaphyseal bone measurements of the non-dominant tibia and bone images were analyzed using the integrated software version 6.00 in this study (Stratec Medizintechnik GmbH, Pforzheim, Germany). Scans were obtained with a voxel size of 0.4 mm, a slice thickness of 2.2 mm, and a scan speed of 20 mm/sec. The 4% tibia total and trabecular bone analyses were performed with the following parameters: Contmode 3, Peelmode 4, trabecular thresholds of 169 mg/cm^3 and 650 mg/cm^3. Contmode 3 used automated contour detection with a user-defined threshold. Peelmode 4 was a threshold driven peel that also utilized a filter. After the initial peel to define cortical and trabecular bone was completed, Peelmode 4 then peeled a set percentage (10%) of the total bone area from the endosteal edge found by the initial peel. This method separated trabecular bone from the cortical + subcortical bone, so as to prevent higher density voxels from being included in the trabecular analysis. The total bone analysis at the 38% and 66% site was performed using threshold driven modes Contour Mode 1 and Peel Mode 2 with a threshold of 710 mg/cm^3. The cortical bone analysis was performed using Cortical Mode 2, a threshold

driven separation mode with a filter, with a threshold of 710 mg/cm^3. When determining SSI, a threshold of 480 mg/cm^3 was used. Muscle CSA analysis was performed as a combination of two analyses. The first trabecular parameter was Contour Mode 3 with Peel Mode 2, using thresholds of -100 mg/cm^3 and 40 mg/cm^3. The second trabecular parameter was Contour Mode 1 and Peel Mode 2, using thresholds of 710 mg/cm^3 and 40 mg/cm^3. Cortical parameters were only utilized for the first analysis, using a threshold of 710 mg/cm^3 in Contour Mode 1. Smoothing filter F03F05 was used. Muscle CSA at the tibia 66% site was defined with the following equation: Subcortical area (Analysis 1) – Cortical Area (Analysis 1). The cone phantom calibration was performed at the beginning of each testing day prior to the participant being scanned, and the cortical calibration was performed every 7 days.

Non-dominant tibia length was measured by a tape measure in "mm" prior to scanning. When measuring the tibia, the participant sat on a chair and crossed her non-dominant leg over the other knee. The length of the tibia was measured from the end of the medial malleolus at the ankle to the endplate of the tibia plateau at the proximal tibia. A small pen mark was made at these bony landmarks when making measurements.

After entering the participant's basic information in the computer, the participant sat on the chair of the pQCT and put the non-dominant leg on the leg and foot support. The chair position was adjusted to make sure the individual's leg was centered and straight while seated. Straps were put around the foot and knee to stabilize the leg. Then the laser point in the gantry was positioned directly below the pen marker on the ankle, and the Scout View (SV) was obtained first to find the start position for

the tibia scanning. Based on the SV, the reference line was placed at the middle of the "football area" (tibio-talar joint), and the cross-sectional CT scan started obtaining the bone images. Similarly, the SV was assessed first at the distal lateral endplate of the femur to locate the start position for the femur scanning and then started the cross-sectional CT scans at the 50% of the femur length from distal. The participant was required to remain still throughout the whole scanning. All pQCT scans were performed by the same qualified technician.

All participants had their total and trabecular vBMD at tibia 4% site, total and cortical vBMD at tibia 38% and 66% sites, and muscle CSA at tibia 66% sites measured by pQCT. Participants received radiation exposure of an absorbed dose of 1 mrem from each scan, which was almost the amount of radiation exposure that Americans receive in one day from natural background radiation (300mrem/year) from sources such as radioactivity in the soil.

Precision of volumetric bone variables was assessed by the technician who measured all the participants on PQCT. Similarly, the coefficient of variation (CV%) was calculated using the ISCD Bone Densitometry Precision Calculating Tool, and the results are shown in Table 2. Overall, the precision for measuring volumetric bone variables in postmenopausal women ranged from 0.29% to 3.07% depending on the site and variable.

Table 2. Precision for Volumetric Bone Measurements by PQCT

Site	Variable	CV%
4%	Total vBMD	1.12%
	Total vBMC	2.00%
	Trabecular vBMD	0.68%
	Trabecular vBMC	3.07%
	Peri_C	1.23%
38%	Total vBMD	0.29%
	Total vBMC	0.48%
	Cortical vBMD	0.29%
	Cortical vBMC	0.61%
	SSI	1.29%
	Iploar	0.84%
66%	Total vBMD	1.51%
	Total vBMC	0.92%
	Cortical vBMD	0.50%
	Cortical vBMC	1.85%
	Peri_C	0.49%
	Endo_C	1.45%
	SSI	1.49%
	Iploar	1.08%
	Muscle CSA	1.73%

Peri_C: Periosteal Circumference; Endo_C: Endosteal Circumference; SSI: Stress Strain Index; Muscle CSA: Muscle Cross-sectional Area

Muscle Functional Performance Measurements

Handgrip Test

Grip strength was measured using a handgrip dynamometer (Takei Scientific Instruments, Yashiroda, Japan) while in seated position. The participant flexed her elbow at 90 degrees with the forearm in a neutral position and the wrist between 0-30 degrees dorsiflexion, and 0-15 degrees ulnar deviation in the dominant hands. The Takei dynamometer was used first. The grip width was adjusted so that the instrument felt comfortable to squeeze in the hand. Once in position, starting from the right hand, the participant was encouraged to squeeze as hard as possible or until the needle stopped rising for about 3-5 seconds. The same measurement was repeated in the left hand and two further measurements for each hand alternating sides to give 3 readings in each side. The results were recorded to the nearest 0.1 kg and the maximal handgrip strength was used in analyses.

Gait Speed Test

Gait speed was measured using an 8-meter straight path marked with tape. Any usual walking aids, such as cane or walker, were allowed in the test. After the tester's command of "Go", the participant started to walk at their usual pace along the path. Digital stopwatch was used to record the time. Three repetitions were performed and a minimum of 1 min rest between trials or as long as the participant needed was given. Trained testers supervised the testing process. The average time of three repetitions was used to calculate the gait speed (distance/time).

Jump Test

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). Farias et al. (36) reported that the contact mat provided a reliable vertical jump power in older women (ICC=0.91). Rogan et al. (110) found strong correlations between jump mat and force plate results in terms of jump height (r=0.99) and ground contact time (r=0.98) in young adults. The participant in our study was asked to do a countermovement vertical jump by crouching, then jumping with non-restricted arm motion, and then landing on the jump mat. Trained spotters were standing on either side of the participant to help with balance, if needed. A transfer belt was fastened around the waist of the participant and was held by the spotter to stabilize the participant if she lost her balance. A minimum of 1 min or as long as the participant needed was allowed between jumps, and 3 successful jumps were performed by each participant. The average performance of the three trials was used in the data analysis.

The test-retest reliability of functional performance tests (grip strength, gait speed, and jump test) in postmenopausal women was assessed by the technician who tested all the participants. The values are shown in Table 3. The intraclass correlation (ICC) ranged from 0.78 to 0.94 and the Pearson *r* ranged from 0.81 to 0.94. Overall, these tests demonstrated good test-retest reliability in postmenopausal women.

Table 3. Test-retest Reliability of Functional Performance Measurements

Variable	Day 1	Day 2	ICC (3,1)	Pearson *r*
Grip Strength (kg)	22.6 ± 4.2	22.7 ± 4.9	0.874	0.885
Gait Speed (m/s)	1.22 ± 0.15	1.24 ± 0.15	0.821	0.821
JHt (inch)	5.9 ± 1.7	5.8 ± 1.7	0.942	0.942
JVel (m/s)	0.85 ± 0.16	0.88 ± 0.13	0.784	0.805
JP (W)	613.3 ± 141.3	638.2 ± 131.9	0.839	0.841

JHt: Jump Height; JVel: Jump Velocity; JP: Jump Power; Day 1 and Day 2 were reported as Mean ± SD; ICC: Intraclass Correlation

Sarcopenia Classification

Sarcopenia status in postmenopausal women was determined based on the conventional definition as well as the criteria set by EWGSOP (23). Conventional definition: skeletal muscle mass index (SMI) < -2 SD 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.

Blood Sampling

A blood sample of 7.5 ml was collected via venipuncture by a registered nurse or phlebotomist at the OU Goddard Student Health Center in the early morning between 8:00 a.m. and 9:00 a.m. after an 8-hour overnight fasting to measure levels of c-miRNAs and BTMs. After the blood sample was drawn, it was allowed to clot for at least 30 min and then centrifuged at 2,000 g for 15 min. Following that, serum samples were transported to the PI's laboratory, aliquoted into 10 microtubes, and immediately frozen at -84 °C until analyzed.

Biochemical Markers of Bone Turnover

Prior to the assay, frozen samples along with all reagents were thawed at room temperature. Levels of bone resorption markers, CTX and TRAP5b, and bone formation marker, P1NP were measured in duplicate using the ELISA kits. The CTX (Immunodiagnostic Systems, Gaithersburg, MD) and TRAP5b (Quidel, Athens, OH) assays were performed following step by step kit instructions (Appendices E & F). The intra-assay CV% were 1.3-15.2%, and the inter-assay CV% were 1.3-7.0%. So far, most of the P1NP assays are performed using automated immunoassay systems, which was not available in our laboratory. We tried two manual immunoassay kits from two companies (US Biological, Salem, MA and Cloud-Clone, Wuhan, China), but the P1NP results were inconsistent thus were not used in data analysis.

Selection of Candidate MiRNAs

Three muscle-specific miRNAs and five bone-specific miRNAs (miR-1-3p, -21-5p, -23a-3p, -24-3p, -100-5p, -125b-5p, -133a-3p), were selected as representatives of those previously implicated underlying cellular processes (Table 4). For simplicity, they are referred to as miR-1, -21, -23a, -24, -100, -125b, -133a throughout the chapters. Note that some of these miRNAs regulate several functions. For example, miR-21 has been reported to be associated with both osteoporosis and fractures (116) and sarcopenia in the elderly (17). MiR-133a not only regulates myogenesis, but it is also a potential biomarker for osteoporosis (134).

Table 4. 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 (123)
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

RNA Extraction

Total RNA was extracted using the miRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) following the step by step procedures in the kit instructions (Appendix G). Serum samples were thawed at room temperature. 200 µL of serum was used for sample lysis by mixing with 1000 µL QIAzol Lysis Reagent. RNA extraction was performed using 200 µL chloroform and phase separation was achieved by centrifugation for 15 min at 12,000 g at 4°C**.** 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). Spin columns were washed with RWT buffer, RPE buffer and 80% ethanol. In the last step, 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): Selected the RNA app, first established a Blank using 1 µL of RNase-free water, then measured the RNA concentration using 1 µL of extracted RNA. The full spectrum analysis was generated, providing the absorbance values at 260 nm (A260) and the ratio of the 260 nm and 280 nm (A260/A280). To ensure significance, A260 readings should be greater than 0.15. A260/A280 gives an estimate of RNA purity, with readings of 1.9-2.1 indicating pure RNA. RNA concentration results were reported in ng/µL. Extracted RNA samples were immediately frozen in a -80°C freezer. Two-step quantitative reverse-transcription PCR (qRT-PCR) was used for RNA quantification: first of all, cDNA synthesis (reverse transcription), and then about 10% of cDNA is transferred for real-time PCR.

cDNA Synthesis

The extracted total RNA was transcribed to cDNA using the TaqMan Advanced miRNA cDNA Synthesis Kit (Applied Biosystems, Foster City, CA) via a TC-412 Thermal Cycler (Techne, UK). Starting with a total RNA sample, poly(A) polymerase was used to add a 3'-adenosine tail to the miRNA. Then the miRNA with poly(A) tail underwent adaptor ligation at the 5' end. The adaptor acts as the forward-primer binding site for the miR-Amp reaction. Therefore, all miRNAs were amplified in a single reverse transcription (RT) reaction. Then a universal RT primer bound to the 3' poly(A) tail and the miRNA was reverse transcribed. During miR-Amp reaction, universal

forward and reverse primers increase the number of cDNA molecules. The kit instructions are listed in Appendix H (Section 2).

Total RNA and cDNA synthesis reagents were thawed to room temperature on ice. 2 µL of total RNA were mixed with 3 µL Poly(A) Reaction Mix in PCR 8-tube strips to perform polyadenylation in thermal cycler for 55 min. Then 10 µL of the Ligation Reaction Mix was added to each tube containing the poly(A) tailing reaction product, and legation was performed in thermal cycler for 60 min. Following that, 15 µL of the Reverse Transcription Reaction Mix was added to each tube containing the adaptor ligation reaction product, and Reverse Transcription (RT) was performed in the thermal cycler for 20 min. Finally, 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. Eventually, a total volume of 50 µL miR-Amp reaction product of each sample was frozen in -80°C freezer immediately.

Quantification of MiRNA Expression

TaqMan Advanced miRNA assays (Applied Biosystems, Foster City, CA) and quantitive real-time Polymerase Chain Reaction (qPCR) were used to quantify the relative expression levels of selected c-miRNAs. TaqMan-based detection uses a fluorogenic probe specific to a target gene to detect the target. The TaqMan MGB probes contain a reporter dye at the 5’ end of the probe and a non-fluorescent quencher (NFQ) dye at the 3’ end of the probe. The NFQ dye does not fluoresce, which allows the real-time PCR to measure the reporter dye contributions more accurately. During

the PCR, the forward and reverse primers anneal to the complementary sequences along the denatured cDNA template strands. The TaqMan MGB probe anneals specifically to a complementary sequence between the forward and reverse primer sites. During polymerization, the DNA polymerase only cleaves probes that hybridize to the target sequence. Cleavage separates the reporter dye from the probe, which results in increased fluorescence by the reporter dye. The kit instructions are provided in Appendix H (Section 3).

Each 10 µL miR-Amp reaction was diluted with 180 µL 0.1X TE buffer (1:10) to prepare 200 µL of diluted cDNA template, and then 100 µL of each was aliquoted into PCR 8-tube strips and kept in -20°C freezer. 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 sealed with MicroAmp Optical Adhesive Film (Applied Biosystems, Foster City, CA) and the contents briefly spun down using MPS 1000 Mini PCR Plate Spinner (Labnet International, Woodbridge, NJ) to eliminate air at the bottom of wells. 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 with the following settings: 50°C for 2 min, 95°C for 2 min (Enzyme activation), 45 cycles of 95°C for 1 s (Denaturation) and 60°C for 20 s (Annealing/Extension).

miR-16-5p, -93-5p, and -191-5p were selected as endogenous controls based on previous literature. 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 (19). MiRNAs with $Ct \geq 37$ were considered not expressed.

Data Analyses

Statistical analyses were performed using IBM SPSS Statistics (SPSS Inc., Chicago, IL), version 22. The relative expression levels of c-miRNA are reported as mean ± standard error (SE), and the rest of descriptive data are 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 ($p > 0.05$). However, variables of miRNAs and BPAQ scores were not normally distributed ($p < 0.05$). 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 (23), definitions. Participants were further divided into normal (aBMD T-score > -1 with normal SMI), osteopenia (aBMD T-score ≤ -1 with normal SMI), sarcopenia (aBMD T-score > -1, SMI ≤ 5.5 kg/m^2) and sarco-osteopenia (aBMD T-score ≤ -1, SMI ≤ 5.5 kg/m^2) groups. However, there only one participant fell into the sarcopenia group, which was excluded from ANOVA analysis.

Chi-square analyses were used to examine the associations between the prevalence of osteoporosis and sarcopenia.

Independent samples t-test was used to compare physical characteristics, bone and muscle variables, functional performance between two groups (normal/osteoporosis, normal/sarcopenia), whereas one-way ANOVA was used to compare them among normal, osteopenia, and sarco-osteopenia 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, -21, -23a, -24, -100, -125b, -133a, -206) in osteoporosis group alone, sarcopenia group alone, or osteopenia and sarco-osteopenia groups were compared to that of the normal group. Two-tailed Mann-Whitney U tests were used to compare the relative expression levels of c-miRNAs between normal and osteoporosis groups, and normal and sarcopenia groups, whereas two-tailed Kruskal-Wallis tests were used to compare the relative expression levels of c-miRNAs among normal, osteopenia, and sarco-osteopenia 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 $p \leq 0.05$.

Chapter 4 Results and Discussion

The purpose of this study was to identify specific c-miRNAs that are associated with bone and muscle status in postmenopausal women, and to evaluate the use of these c-miRNAs as biomarkers of osteoporosis and sarcopenia. In addition, this study examined the associations between specific c-miRNAs and bone turnover markers as well as sarcopenia parameters.

Participant Characteristics

A total of 123 postmenopausal women aged 60 to 85 years old were screened for this study. Ninety-nine participants met the pre-screening criteria and signed the consent form. Of the 99 participants, 24 participants were excluded from the study for the following reasons: 6 due to time conflicts, 12 having hypertension, 2 taking antidepressants drug SSRI (Selective Serotonin Reuptake Inhibitors), another 2 taking thiazide medication, 1 due to recent fractures within 12 months, and another 1 because the phlebotomist was not able to get blood draw from her. Therefore, a total of 75 postmenopausal women aged 60 to 85 years old met all the inclusion criteria and completed the study in the Bone Density Laboratory. Among these 75 participants, only one participant was Asian and the rest of them were Caucasian. 31 participants had taken HRT (hormone replacement therapy) in the past, 19 participants were currently taking HRT (7 months to 45 years), and 5 participants were on bisphosphonate treatment, including 3 participants taking both HRT and bisphosphonate treatment.

The number of participants and the prevalence of osteoporosis and sarcopenia among the participants are displayed in Table 5. The prevalence of sarcopenia based on the conventional definition was about three times of that based on the EWGSOP

definition. 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. Chi-square analysis showed that there was no significant association between sarcopenia and osteopenia statuses in this group of participants (Table 6). Also, there was no significant association between bone status and current HRT status (Table 7). However, there was a trend for significant positive association ($p = 0.056$) between muscle status and current HRT status (Table 8), which indicates that HRT current users have a potentially higher prevalence of sarcopenia than HRT non-users.

Table 5. Classification of Participants based on Bone and Muscle Status

Criteria	Classification	Number	Percent
aBMD Status	Normal	14	18.7%
	Osteopenia	51	68.0%
	Osteoporosis	10	13.3%
Sarcopenia Status	Normal	59	78.7%
(Conventional)	Sarcopenia	16	21.3%
Sarcopenia Status	Normal	69	92.0%
(EWGSOP)	Sarcopenia	6	8.0%

Table 6. Association between the Bone and Muscle Status

Classification	Sarcopenia (n=16)	Non-sarcopenia (n=59)	*P*
Normal aBMD (n=14)	7.1% (1)	92.9% (13)	
Osteopenia (n=51)	21.6% (11)	78.4% (40)	0.153
Osteoporosis (n=10)	40.0% (4)	60.0% (6)	

Table 7. Association between Bone and Current HRT Status

Classification	HRT Users (n=19)	Non-Users (n=56)	*P*
Normal aBMD (n=14)	35.7% (5)	64.3% (9)	
Osteopenia (n=51)	25.5% (13)	74.5% (38)	0.360
Osteoporosis (n=10)	10.0% (1)	90.0% (9)	

HRT Users: Participants currently taking hormone replacement therapy (HRT) over 6 months

Table 8. Association Between Muscle and Current HRT Status

Classification	HRT Users (n=19)	Non-Users (n=56)	*P*
Normal (n=59)	20.3% (12)	79.7% (47)	0.056
Sarcopenia (n=16)	43.8% (7)	56.3% (9)	

HRT Users: Participants currently taking hormone replacement therapy (HRT) over 6 months

Altogether, 17.3% of participants (NOR, n=13) had normal muscle mass and aBMD, 61.3% of participants (OP, n=46) had osteopenia 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 (NOR, OP, and SOP).

The characteristics of normal, sarcopenia, osteopenia, and sarco-osteopenia groups are provided in Table 9. 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 normal, osteopenia, and sarco-osteopenia groups. However, the sarco-osteopenia group had significantly lower body weight than the normal ($p = 0.005$) and osteopenia ($p = 0.001$) groups.

Table 9. Physical Characteristics of the Participants

Variable	Group			
	NOR (n=13)	**OP (n=46)**	**SOP (n=15)**	**SP (n=1)**
Age (yr)	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 (yr)	19.0 ± 15.0	13.4 ± 10.4	17.3 ± 7.4	45

Mean ± SD; Ca^{++}: Calcium Intake; 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; ** $p < 0.01$ significant difference between NOR and SOP groups; †† $p < 0.01$ significant difference between OP and SOP groups

Table 10 displays body composition for the four groups of participants. The sarco-osteopenia group had significantly lower muscle mass ($p < 0.001$) for the total body, arms, and legs, and fat mass for the arms ($p = 0.025$ and $p = 0.007$, respectively) than the normal and osteopenia groups. In addition, fat mass at the total body ($p = 0.031$) and leg sites ($p = 0.044$) were significantly lower in the sarco-osteopenia group compared to the osteopenia group. Overall, the sarco-osteopenia group had significantly lower (approximately 20%) appendicular bone free lean mass than the normal and osteopenia groups ($p < 0.001$).

Table 10. Body Composition of the Participants

Variable	Group			
	NOR (n=13)	**OP (n=46)**	**SOP (n=15)**	**SP (n=1)**
% 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
Arm %Fat	37.9 ± 7.5	37.6 ± 8.0	33.9 ± 5.8	47.8
Arm BFLBM (kg)	3.9 ± 0.4	3.8 ± 0.5	3.2 ± 0.4***†††	3.2
Arm FM (kg)	2.7 ± 0.9	2.6 ± 0.9	1.8 ± 0.5*††	3.1
Leg %Fat	42.8 ± 7.0	43.4 ± 7.7	41.6 ± 6.0	37.8
Leg BFLBM (kg)	12.9 ± 1.2	12.8 ± 1.7	10.5 ± 1.0***†††	10.3
Leg FM (kg)	10.8 ± 3.8	11.0 ± 3.9	8.3 ± 2.3†	6.7
ASM (kg)	16.8 ± 1.5	16.7 ± 2.1	13.7 ± 1.3***†††	13.5

Mean ± SD; BFLBM: Bone Free Lean Body Mass; FM: Fat Mass; ASM: Appendicular Skeletal Muscle Mass; * $p < 0.05$; *** $p < 0.001$ significant difference between NOR and SOP groups; † $p < 0.05$; †† $p < 0.01$; ††† $p < 0.001$ significant difference between OP and SOP groups

Physical Functional Performance

Table 11 shows the functional performance for the four groups of participants. Compared to the normal group, the sarco-osteopenia group had significantly lower grip strength ($p = 0.008$). In addition, the sarco-osteopenia group had significantly lower (approximate 20%) jump power than the normal and osteopenia 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 the normal, osteopenia, and sarco-osteopenia groups.

Table 11. Functional Performance of the Participants

Variable	Group			
	NOR (n=13)	OP (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; JHt: Jump Height; JVel: Jump Velocity; JP: Jump Power; * $p < 0.05$; ** $p < 0.01$ significant difference between NOR and SOP groups; †† $p < 0.01$ significant difference between OP and SOP groups

Areal Bone Mineral Density (aBMD)

Table 12 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 osteopenia and sarco-osteopenia groups had significantly lower aBMD than the normal group ($p < 0.01$).

Table 12. Areal Bone Mineral Density Measured by DXA

Variable	Group			
	Normal (n=13)	**OP (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 Tot Hip	1.008 ± 0.058	0.881 ± 0.091$^{\#\#\#}$	0.828 ± 0.104***	0.992
Right Tot Hip	0.990 ± 0.060	0.872 ± 0.087$^{\#\#\#}$	0.832 ± 0.103***	0.970

Mean ± SD; $^{\#\#}$ $p < 0.01$; $^{\#\#\#}$ $p < 0.001$ significant difference between NOR and OP groups; ** $p < 0.01$; *** $p < 0.001$ significant difference between normal and SOP groups

Volumetric Bone Variables

Tables 13, 14 and 15 illustrate the volumetric bone density, bone mass, and bone strength at 4%, 38% and 66% of the tibia, respectively. No significant differences in these bone variables were observed between groups, except for cortical vBMC which was significantly lower at 38% of the tibia in the sarco-osteopenia group than the normal group ($p = 0.042$). Furthermore, the sarco-osteopenia group had significantly smaller (approximately 10%) muscle CSA at 66% of the tibia than the normal and osteopenia groups ($p = 0.001$ and $p < 0.001$, respectively). When participants were classified by bone status alone, the osteoporotic group had significantly lower vBMD, vBMC, and BSI at 4% of the tibia than the normal and osteopenia groups ($p < 0.01$). At the 38% and 66% of the tibia, the osteoporotic group had significantly lower total and

cortical vBMC, cortical area, and SSI than the normal and osteopenia groups ($p < 0.05$), and significantly lower total vBMD than the normal group ($p < 0.05$).

Table 13. Volumetric Bone Variables at Tibia 4% Site

Variable	Group			
	NOR (n=13)	**OP (n=46)**	**SOP (n=15)**	**SP (n=1)**
Tot vBMD (mg/cm^3)	288.5 ± 43.7	264.6 ± 33.9	261.8 ± 36.2	319.1
Tot vBMC (mg/mm)	277.2 ± 31.7	262.0 ± 35.9	254.4 ± 43.6	274.3
Tot Area (mm^2)	969.7 ± 82.8	995.9 ± 117.4	973.0 ± 113.7	859.5
Trab vBMD (mg/cm^3)	243.1 ± 38.7	226.8 ± 32.1	232.1 ± 36.8	281.0
Trab vBMC (mg/mm)	191.7 ± 22.7	185.6 ± 30.0	187.8 ± 37.2	197.5
Trab Area (mm^2)	797.1 ± 77.9	822.8 ± 103.9	809.7 ± 100.6	702.9
Peri_C (mm)	110.3 ± 4.5	111.7 ± 6.7	110.4 ± 6.4	103.9
Total BSI (mg^2/mm^4)	81.2 ± 19.9	70.1 ± 16.0	67.7 ± 18.9	87.5
Trab BSI (mg^2/mm^4)	47.3 ± 12.6	42.7 ± 11.3	44.6 ± 14.2	55.5

Mean ± SD; Tot: Total; Trab: Trabecular; Peri_C: Periosteal Circumference; BSI: Bone Strength Index

Table 14. Volumetric Bone Variables at Tibia 38% Site

Variable	Group			
	NOR (n=13)	**OP (n=46)**	**SOP (n=15)**	**SP (n=1)**
Tot vBMD (mg/cm^3)	911.1 ± 66.5	856.9 ± 75.5	857.0 ± 91.2	970.7
Tot vBMC (mg/mm)	311.6 ± 22.4	297.8 ± 34.0	281.2 ± 34.9	306.0
Tot Area (mm^2)	343.7 ± 34.3	348.8 ± 39.5	328.8 ± 29.6	315.2
Cort vBMD (g/cm^3)	1175.4 ± 24.9	1153.6 ± 34.9	1166.0 ± 33.9	1200.3
Cort vBMC (mg/mm)	296.9 ± 23.6	282.2 ± 35.2	264.5 ± 37.4*	295.6
Cort Area (mm^2)	252.6 ± 19.5	244.7 ± 31.0	226.6 ± 29.3	246.2
Peri_C (mm)	65.6 ± 3.3	66.1 ± 3.7	64.2 ± 2.9	62.9
Endo_C (mm)	33.5 ± 4.9	35.8 ± 5.0	35.6 ± 4.5	29.4
Ipolar (mm^4)	19608 ± 3397	19531 ± 4152	17143 ± 2937	16909
SSI (mm^3)	1409.0 ± 184.8	1387.8 ± 223.8	1277.5 ± 168.5	1249

Mean ± SD; Tot: Total; Cort: Cortical; Peri_C: Periosteal Circumference; Endo_C: Endosteal Circumference; SSI: Stress Strain Index; * $p < 0.05$ significant difference between NOR and SOP groups

Table 15. Volumetric Bone Variables at Tibia 66% Site

Variable	Group			
	NOR (n=13)	**OP (n=46)**	**SOP (n=15)**	**SP (n=1)**
Tot vBMD (mg/cm^3)	670.5 ± 67.6	611.1 ± 86.8	636.0 ± 91.5	744.3
Tot vBMC (mg/mm)	337.2 ± 29.1	316.0 ± 40.6	305.0 ± 45.7	339.7
Tot area (mm^2)	506.5 ± 54.3	523.0 ± 69.6	481.3 ± 46.0	456.5
Cort vBMD (g/cm^3)	1122.7 ± 23.8	1098.9 ± 36.5	1116.9 ± 32.1	1156.5
Cort vBMC (mg/mm)	302.0 ± 28.9	275.3 ± 43.2	267.3 ± 49.9	314.7
Cort area (mm^2)	268.9 ± 24.6	250.1 ± 36.4	238.8 ± 40.8	272.2
Peri_C (mm)	79.7 ± 4.2	80.9 ± 5.4	77.7 ± 3.7	75.7
Endo_C (mm)	54.3 ± 5.8	58.1 ± 7.5	54.9 ± 5.8	48.1
Ipolar (mm^4)	36205 ± 6080	35505 ± 7591	30709 ± 5604	31569
SSI (mm^3)	2067.8 ± 225.0	2023.1 ± 332.1	1856.3 ± 296.4	1916.4
Muscle CSA (mm^2)	6205.5 ± 469.0	6145.4 ± 721.6	$5300.5 \pm 481.6^{**\dagger\dagger\dagger}$	4437.3

Mean ± SD; Tot: Total; Cort: Cortical; Peri_C: Periosteal Circumference; Endo_C: Endosteal Circumference; SSI: Stress Strain Index; Muscle CSA: Muscle Cross-sectional Area; ** $p < 0.01$ significant difference between NOR and SOP groups; ††† $p < 0.001$ significant difference between OP and SOP groups

Bone Turnover Markers

Table 16 shows the concentrations of bone resorption markers CTX and TRAP5b in the four groups. No significant differences in BTMs were found in normal, osteopenia, and sarco-osteopenia groups.

Table 16. Serum Bone Turnover Marker Concentrations

Variable	Group			
	NOR (n=13)	OP (n=46)	SOP (n=15)	SP (n=1)
CTX (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; C/T Ratio: CTX/TRAP5b Ratio

Expression of Circulating MiRNAs (c-miRNAs)

As mentioned in Chapter 3, miRNAs with Ct ≥ 37 were considered not expressed since these expression levels indicated large variability (91). As a result, 3 samples were not expressed in 3 endogenous controls (miR-16, -93, -191), another 3 sample were not expressed in 2 endogenous controls (miR-93, -191), and 6 samples were not expressed in 1 endogenous control (3 of miR-93; 3 of miR-191). In total, 12 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. Unfortunately, even for the 63 samples, only about a third of circulating miR-1 (n=23), -133a (n=14), -100 (n=24) and none of circulating miR-206 were expressed. Therefore, the relative expression levels of the rest of the target miRNAs (miR-21, -23a, -24, -125b) between the 4 groups, which are all bone-specific miRNAs, are reported in Table 17 and Figure 1 (A-D). In the boxplots shown in Figure 1, the central lines indicate the median, boxes reflect first and third quartiles, and error bars indicate minimum and maximum values. Biologically, fold change of greater than 2 (corresponding to a $\log_2$-fold change of 1) is considered upregulation, whereas fold change of lower than 0.5 (corresponding to a $\log_2$-fold change of -1) is considered as

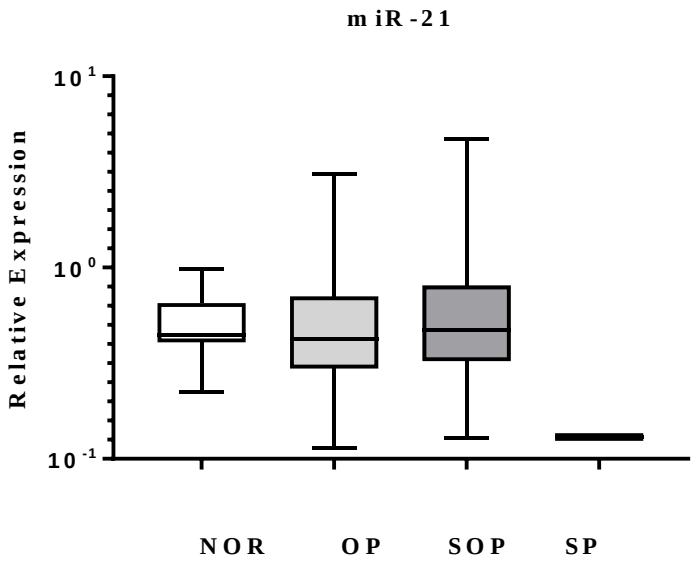
downregulation (55). In our study, fold changes ranged from 0.83 to 1.81, and no statistical differences were found in the relative expression levels of these miRNAs (miR-21, -23a, -24, -125b) among normal, osteopenia, and sarco-osteopenia groups.

Table 17. Relative Expression Levels of miRNAs based on Muscle and Bone Status

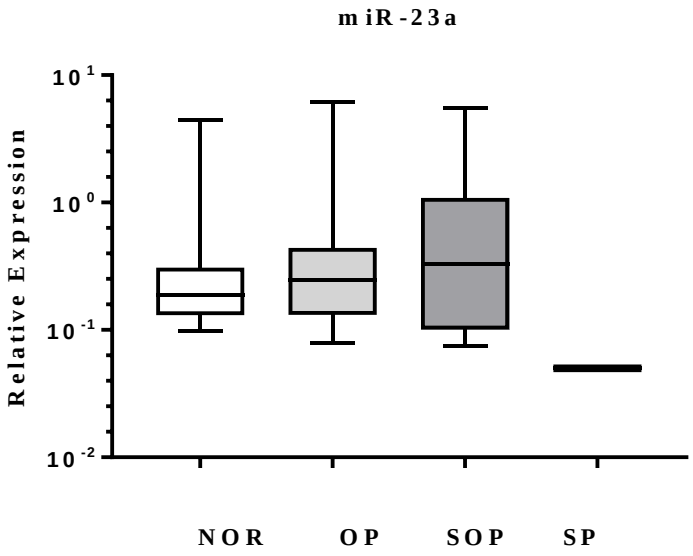
miRNA	**Relative Expression**				**Fold Change**		***p***
	NOR (n=12)	**OP (n=39)**	**SOP (n=11)**	**SP (n=1)**	**1**	**2**	
miR-21 n=62	0.51 ± 0.06 n=12	0.67 ± 0.10 n=39	0.93 ± 0.43 n=10	0.13 n=1	1.31	1.81	NS
miR-23a n=61	0.57 ± 0.36 n=12	0.59 ± 0.21 n=37	1.05 ± 0.49 n=11	0.05 n=1	1.03	1.85	NS
miR-24 n=62	1.89 ± 1.05 n=12	1.57 ± 0.31 n=39	2.31 ± 0.74 n=10	0.46 n=1	0.83	1.22	NS
miR-125b n=56	0.60 ± 0.31 n=12	0.66 ± 0.14 n=33	0.53 ± 0.21 n=10	0.80 n=1	1.09	0.87	NS

Mean ± SE; Fold Change 1: Comparison of OP group to NOR group; Fold Change 2: Comparison of SOP group to NOR group; NS: No significant differences

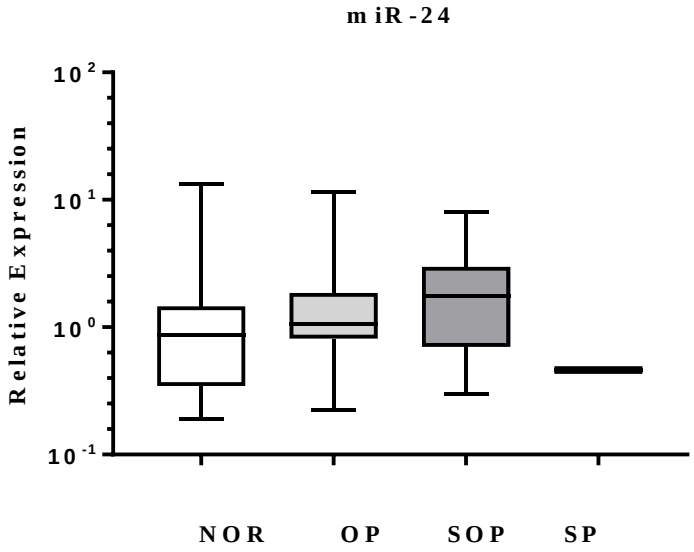
A


miR-21
Relative Expression
10 1
10 0
10 -1
NOR
OP
SOP
SP

B


miR-23a
Relative Expression
10 1
10 0
10 -1
10 -2
NOR
OP
SOP
SP

C



D

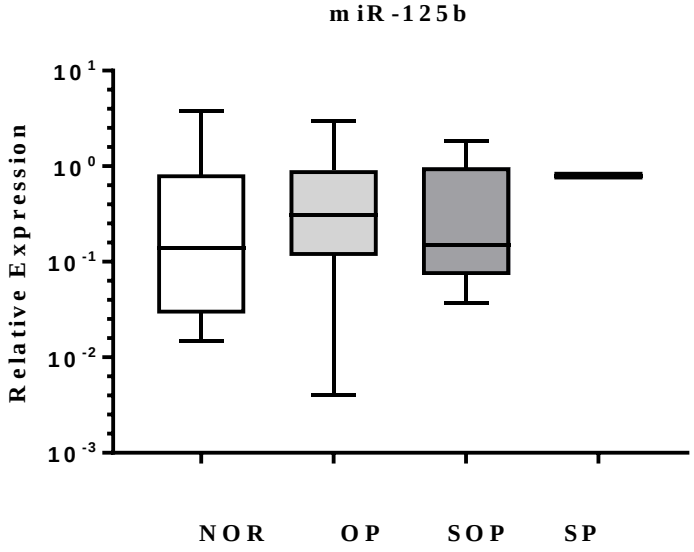


Figure 1. Relative Expression Levels of miRNAs Based on Muscle and Bone Status (Mean ± SE)

Participants were further compared based on muscle status and bone status separately. When comparing the sarcopenia and normal groups, no significant differences were observed in the relative expression levels of c-miRNAs (miR-21, -23a, -24, -125b) between the two groups and fold changes ranged from 0.86 to 1.66 (Table 18). Similarly, no significant differences were found in the relative expression levels of c-miRNAs (miR-21, -23a, -24, -125b) between normal and osteoporosis groups (Table 19). However, fold changes of miR-21 (FC=2.59) and miR-23a (FC=2.09) indicated upregulation in osteoporosis compared to normal, whereas fold change of miR-125b (FC=0.46) indicated downregulation in osteoporosis compared to normal.

Table 18. Relative Expression Levels of miRNAs in Sarcopenia

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

Mean ± SE; NS: No significant difference

Table 19. Relative Expression Levels of miRNAs in Osteoporosis

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

Mean ± SE; NS: No significant difference

Table 20 compares the relative expression levels of miRNAs between HRT current users and non-users. Since there was a reasonable number of expressed miR-1 and miR-100 samples in HRT users, comparisons were made in miR-1 and -100 as well. No statistical differences in relative expression levels were found between the HRT users and non-users groups. Fold changes ranged from 0.23 to 1.57, indicating downregulation of miR-1 (FC=0.23) in the HRT users compared to the non-users.

Table 20. Relative Expression Levels of miRNAs based on HRT Status

miRNA	**Relative Expression**		**Fold**	***p***
	Non-users (n=48)	**HRT Users (n=16)**	**Change**	
miR-1 (n=23)	57.15 ± 44.00 (n=16)	13.07 ± 6.18 (n=7)	0.23	NS
miR-21(n=62)	0.73 ± 0.12 (n=47)	0.51 ± 0.08 (n=15)	0.70	NS
miR-23a (n=61)	0.66 ± 0.19 (n=46)	0.64 ± 0.35 (n=15)	0.96	NS
miR-24 (n=62)	1.68 ± 0.36 (n=47)	1.89 ± 0.55 (n=15)	1.13	NS
miR-100 (n=24)	0.05 ± 0.03 (n=19)	0.05 ± 0.03 (n=5)	1.19	NS
miR-125b (n=56)	0.55 ± 0.13 (n=42)	0.86 ± 0.22 (n=14)	1.57	NS

Mean ± SE; NS: No significant difference

c-miRNA and Age

Table 21 shows the relationships between the relative expression levels of c-miRNAs and age. Specifically, the relative expression level of circulating miR-125b was significantly positively associated with age (r = 0.334, $p = 0.012$) (Figure 2).

Table 21. Spearman Correlations between c-miRNAs and Age

miRNA	r	*p*
miR-1 (n=23)	0.186	0.396
miR-21 (n=62)	0.048	0.713
miR-23a (n=61)	0.083	0.525
miR-24 (n=62)	0.099	0.446
miR-100 (n=24)	0.040	0.854
miR-125b (n=56)	0.334	0.012*
miR-133a (n=14)	-0.011	0.970

* $p < 0.05$, significant Spearman Correlation Coefficient

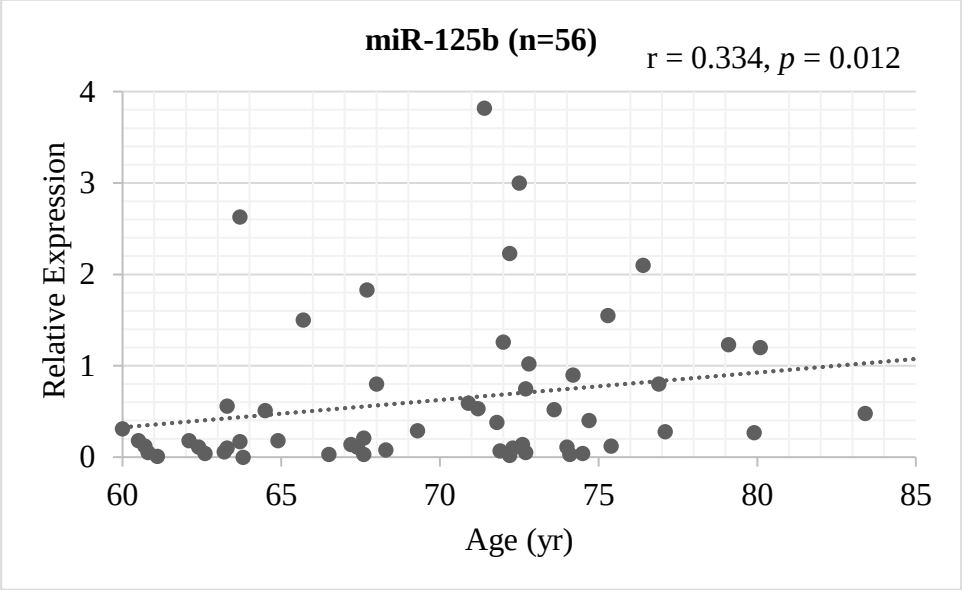


Figure 2. Correlation between Expression Level of miR-125b and Age

c-miRNA and Bone Variables

Spearman correlations showed that the relative expression levels of miR-21 were significantly negatively correlated to the left trochanter BMC (r = -0.252, p = 0.048) (Figure 3), right trochanter BMC (r = -0.294, p = 0.020) (Figure 4), and cortical vBMD at tibia 38% site (r = -0.253, p = 0.047) (Figure 5). There also was a trend for a significant association between the relative expression of miR-21 and lumbar spine aBMD (r = -0.249, p = 0.051). Furthermore, the relative expression level of miR-23a was found to be significantly positively correlated to the level of TRAP5b (r = 0.259, p = 0.044) (Figure 6). Although only 23 samples were expressed in circulating miR-1, the relative expression level of miR-1 was significantly negatively correlated to cortical vBMD at 38% tibia (r = -0.434, p = 0.039). Also, there were trends for significant correlations between the relative expression of miR-1 and cortical vBMD at 66% tibia (r = -0.379, p = 0.074), and cortical area at 38% tibia (r = 0.395, p = 0.062). The relative expression level of miR-133a was positively associated with total body BMC (r = 0.594, p = 0.025), although only 14 samples were expressed in circulating miR-133a. However, no significant correlations were found between other miRNAs (miR-24, -100, -125b) and bone variables.

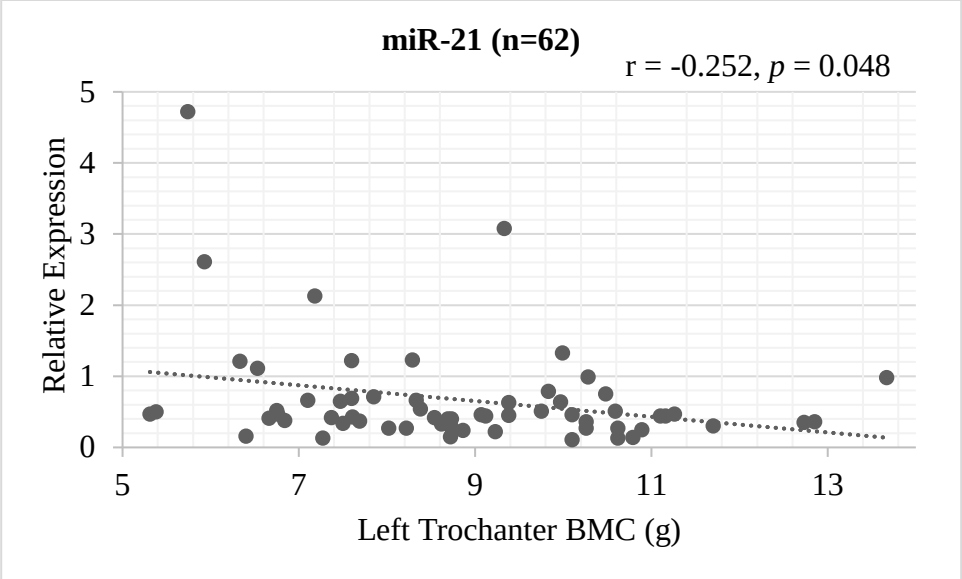


Figure 3. Correlation between Expression Level of miR-21 and Left Trochanter BMC

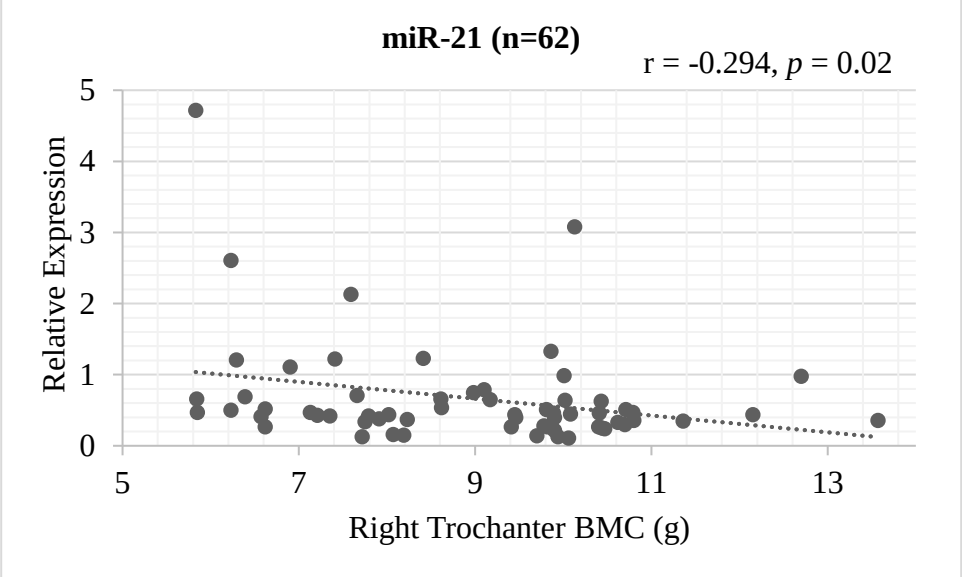


Figure 4. Correlation between Expression Level of miR-21 and Right Trochanter BMC

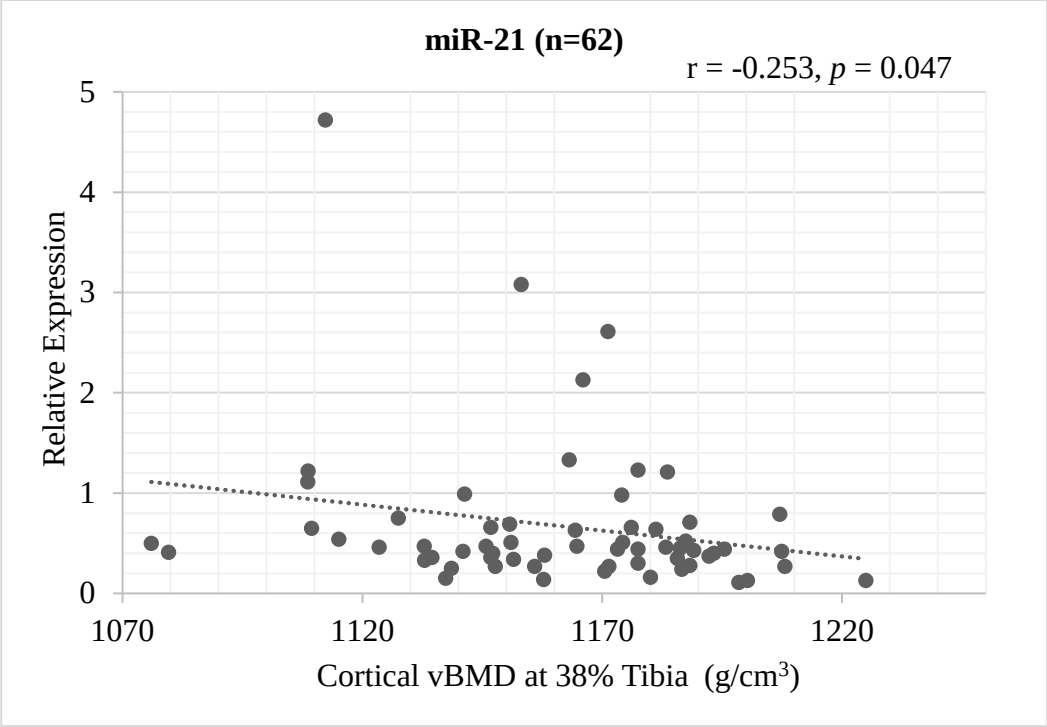


Figure 5. Correlation between Expression Level of miR-21 and Cortical vBMD at 38% Tibia

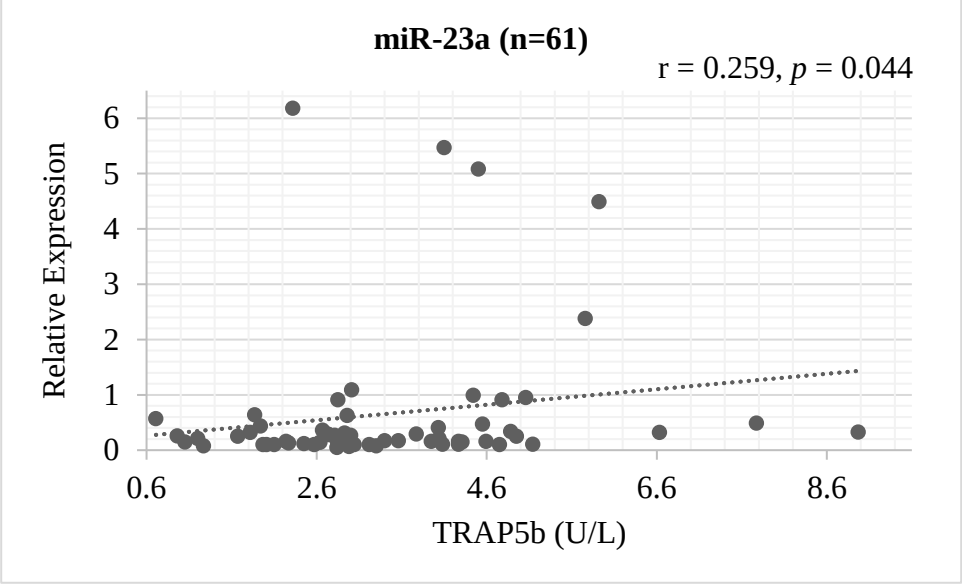


Figure 6. Correlation between Expression Level of miR-23a and TRAP5b Levels

c-miRNA and Muscle Variables

The relative expression level of miR-125b was significantly positively correlated with jump velocity (r = 0.263, p = 0.05) and relative jump power (r = 0.294, p = 0.028) (Figures 7 and 8). However, none of the muscle-specific circulating miRNAs (miR-1, -133a) were correlated with muscle mass, muscle strength, or muscle power.

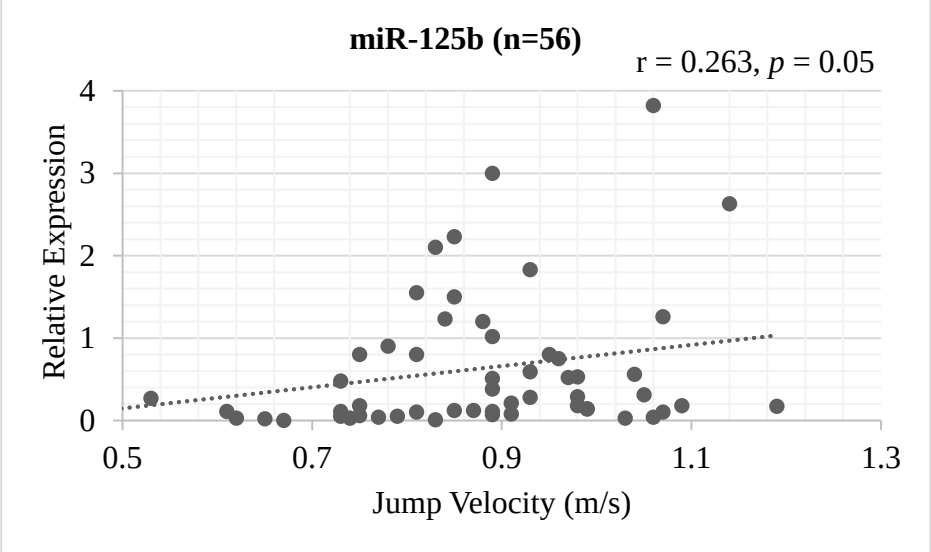


Figure 7. Correlation between Expression Level of miR-125b and Jump Velocity

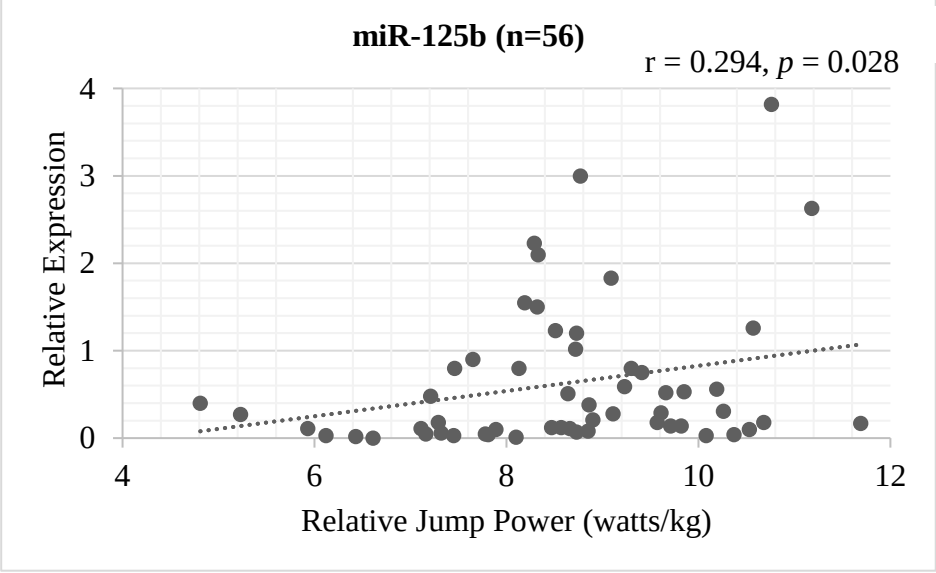


Figure 8. Correlation between Expression Level of miR-125b and Relative Jump Power

Discussion

The current study explored circulating miRNAs (c-miRNAs) that are associated with osteoporosis and sarcopenia in postmenopausal women aged 60 to 85 years. Aging is accompanied with progressive decline in bone density and muscle mass, known as osteoporosis and sarcopenia, respectively. Both osteoporosis and sarcopenia increase the risk of falls and fractures and decrease quality of life, which is a great burden to society. MiRNAs are short, non-coding RNA molecules that fine tune at least 30% of gene expression in humans (129). Recently, circulating miRNAs have been detected and shown to be stable in body fluids (e.g. blood), which have significant clinical potential as disease-specific biomarkers (70). Although many miRNA studies have focused on cancer and cardiovascular diseases, the role of circulating miRNAs in bone diseases is becoming more and more recognized (50, 91). The skeletal muscle is enriched with muscle-specific miRNAs, such as miR-1, -133a/-133b, -206, which have been thoroughly studied in the literature. However, few studies have examined circulating miRNAs and muscle characteristics.

This study compared serum levels of five bone-specific miRNAs (miR-21, -23a, -24, -100, -125b) and three muscle-specific miRNAs (miR-1, -133a, -206) in normal, osteopenic/osteoporotic, sarcopenic, sarco-osteopenic postmenopausal women. To our knowledge, this is the first study examining c-miRNAs in sarcopenia and sarco-osteopenia. The primary findings were that sarco-osteopenia individuals were at the greatest risk of low muscle mass (muscle CSA at 66% of the tibia) and poor functional performance (grip strength) compared to normal and osteopenia individuals. Circulating miR-21 level was negatively associated with trochanter BMC and cortical vBMD at

38% of the tibia, whereas circulating miR-23a level was positively associated with bone resorption marker, TRAP5b. In addition, circulating miR-125b 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 and jump velocity and relative jump power.

This study was designed based on the findings of Seelinger et al. (116), who measured miRNAs in serum and bone tissues in osteoporotic patients and nonosteoporotic controls using microarrays. They found five miRNAs, miR-21, -23a, -24, -100, and -125b, that were significantly upregulated in osteoporotic fracture patients in both serum and bone tissue compared to controls, and these miRNAs were used in our study. In addition, three muscle-specific miRNAs in humans, miR-1, -133, and -206, regulating muscle development, and proliferation and differentiation of myoblasts (117), were used in our study. It is recognized that miR-1 and -133a are highly expressed in cardiac and skeletal muscle, whereas miR-206 is primarily expressed in slow twitch fibers in skeletal muscle (69).

Osteoporosis and Sarcopenia Prevalence

In this study, the prevalence of osteoporosis (aBMD T-score $\leq$ -2.5) was 13% in postmenopausal Caucasian women aged 60 to 85 years. We found that the osteoporotic group had significantly lower vBMD, vBMC, and bone strength at most sites of the tibia compared to the normal and osteopenia groups. According to a population-based study in Minnesota, the prevalence of osteoporosis was approximately 21.6% in women in their 60s, 38.5% in their 70s, and as high as 70% in women over age 80 yrs (59).

Thus, the prevalence of osteoporosis in our study is lower than their prevalence. The discrepancy in findings was probably due to having relative healthy participants in our study since we excluded participants with diabetes or uncontrolled hypertension, taking antidepressants or glucocorticoids, having recent fractures within 12 months, or having metal implants at hip or spine. In addition, our sample size was much smaller than this previous study.

The primary clinical consequence of osteoporosis is fracture, and postmenopausal women are at the greatest risk of fractures. It is estimated that the lifetime risk of fractures at overall sites was around 40% for U.S. Caucasian women, and 13% for U.S. Caucasian men (59). According to the Study of Osteoporotic Fractures, osteoporosis at spine or hip contributed to 8-44% of fractures, and the risk of fractures was about four-fold in osteoporotic patients compared to those with normal aBMD (124). Estrogen plays an important role in regulating bone turnover rate. During the menopausal transition, serum estradiol levels decrease by 90%, resulting in the net imbalance between bone formation and bone resorption, which causes accelerated bone loss, particularly in trabecular bone (40, 103). However, in this study we did not find significant associations between current HRT use and osteopenia/osteoporosis status, probably due to the wide range of the duration of HRT use (7 months to 45 years).

In this study, 21% participants were classified as sarcopenic based on the conventional definition. This percentage is slightly lower than the prevalence of sarcopenia in the New Mexico Elder Health Survey, which reported that 23.1-43.2% of non-Hispanic white females had sarcopenia using the conventional definition (9). The new definition of sarcopenia by the EWGSOP combines low muscle mass and poor

functional performance, which generates a smaller prevalence of sarcopenia. For example, the prevalence of sarcopenia in this study was reduced to 8% based on the EWGSOP definition. According to the International Sarcopenia Initiative (24), the global prevalence of sarcopenia ranges from 1% to 29% in community dwelling elderly using the EWGSOP definition depending on various factors such as gender, age, ethnicity, country. One of the challenges in sarcopenia is the lack of consensus about the operational definition of sarcopenia and the evaluation of muscle strength and function among consensus groups and studies. Some other definitions including the International Working Group (IWG) (2009), the Foundation for the National Institutes of Health (FNIH) (2014), and Asian Working Group for Sarcopenia (AWGS) (2014), all of which use different criteria and cutoff points, making it difficult to compare between studies.

Currently, no drug therapies are recommended to treat sarcopenia, and the management of sarcopenia is based on improving physical activity and diet. Exercise, especially resistance exercise, increases muscle mass and strength, improves quality of life, and prevents fragility and disability. Therefore, exercise is an effective intervention in sarcopenic older adults (109). The review article by Cruz-Jentoft et al. (24) found that 3-18 months of resistance training alone significantly increased muscle mass, strength and functional performance, such as gait speed and chair rise.

Sarco-Osteopenia

In this study, no significant association was found between sarcopenia status and osteopenia/osteoporosis status (aBMD T-score $\leq$ -1.0). However, 20% of the participants had both sarcopenia and osteopenia/osteoporosis, known as “sarco-

osteopenia" (11). Compared to the normal and osteopenia groups, the sarco-osteopenia group had significantly lower body weight, jump power, and muscle CSA at 66% of the tibia. Additionally, the sarco-osteopenia group had significantly lower grip strength and cortical vBMC than the normal group.

There is an increased risk of fractures with advancing age. Fractures are not only due to bone loss, but also associated with other non-skeletal factors, such as gait instability and muscle weakness. A major non-skeletal factor that contributes to the risk of fracture is falls (127). In fact, over 90% of all fractures occur after a fall (26). Gait instability is the major intrinsic risk factor of falls, which is primarily due to muscle weakness, or sarcopenia. Therefore, fall prevention is the key to prevent fractures, and sarcopenia should be the next focus in fracture prevention (11). Huo et al. (58) assessed bone density, functional performance, history of falls and fractures, and nutritional status in a large number of older adults (n=680, mean age=79 yr), which had 41% of sarco-osteopenia. They found that the sarco-osteopenic group had the highest self-reported prevalence of falls and fractures than the other groups (normal, osteopenia/osteoporosis alone, and sarcopenia alone).

Exercise, particularly resistance exercise, prevent fractures in two ways. First, exercise increases muscle mass and strength and improves balance, which further prevents falls. Second, long-term exercise increases BMD and bone strength, which prevents fractures. Nutrition is another important aspect in preventing fractures. The same study by Huo et al. (58) reported that some nutritional factors, such as levels of hemoglobin and serum folate, were associated with sarco-osteopenia status, therefore, nutritional considerations are important in fall and fracture prevention in older adults.

miRNAs and Osteoporosis

In this study, we did not find significant statistical differences in levels of c-miRNAs based on osteoporosis and sarcopenia statuses. However, fold changes of miR-21 (FC=2.59) and miR-23a (FC=2.09) indicated upregulation in the osteoporosis group, whereas fold change of miR-125b (FC=0.46) indicated downregulation in the osteoporosis group. Seeliger et al. (116) 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, -23a, -24, -100, and -125b. Panach et al. (91) found three c-miRNAs, miR-122, -125b, and -21, 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.

We found that the relative expression level of miR-21 was significantly negatively correlated with the trochanter BMC and cortical vBMD at tibia 38% site. Also, there was a trend for significant association between expression of miR-21 and lumbar spine aBMD. So far, miR-21 has been one of the most well-studied miRNAs in bone diseases. Particularly, circulating miR-21 has been consistently found to be upregulated in patients with osteoporotic fractures (91, 116). Panach et al. (91) found that the level of miR-21 was correlated with the levels of the bone resorption marker, CTX (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 (41). On the other hand, we know that estrogen inhibits osteoclastogenesis, and recent research has shown that this inhibitory effect is mediated by miR-21 (126). In fact, miR-21 targets on Fas Ligand (FASL) gene, which induces osteoclast apoptosis (126). 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 and bone variables in our findings. The summary is shown in Figure 9.

Figure 9. Function of miR-21 on Osteoclast

In this study, we found a low positive relationship (r = 0.259) between the relative expression level of miR-23a 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 (53). 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 and TRAP5b found in this study may be attributed to their regulatory roles on bone resorption. The summary is shown in Figure 10.

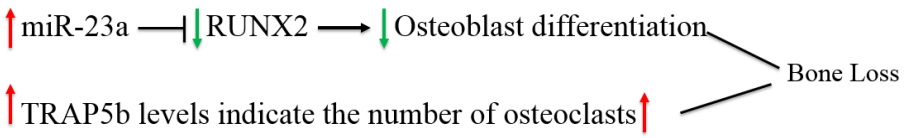


Figure 10. Effects of miR-23a and TRAP5b on Bone

Although many studies have analyzed the role of miRNAs in cancer and cardiovascular diseases, the importance of miRNAs in bone metabolism has been increasingly recognized only recently. Research has indicated that miRNAs are involved in all stages of osteogenesis by regulating differentiation, proliferation, apoptosis, and activity of different bone cells: osteocytes, osteoblasts, osteoclasts, and chondrocytes. Some miRNAs stimulate osteoblastogenesis, such as miR-15b, -20a, whereas some miRNAs inhibit osteoblastogenesis, such as miR-23a, -100, -133a. This occurs primarily through regulating BMP-RUNX2 and Wnt signaling pathways. For example, miR-100 directly targets BMPR2 (BMP receptor type II) and inhibits osteoblastogenesis. Less information is known about the role of miRNAs in osteoclasts than osteoblasts. MiR-21 inhibits PCD4 (programmed cell death 4) protein expression and induces osteoclastogenesis. The role of miRNAs in osteocytes is unknown, however, miRNAs regulate the differentiation and proliferation of chondrocytes. For example, miR-125b is reported to stimulate the differentiation of chondrocytes in mouse by repressing an inhibitor of chondrogenesis (84).

Since miRNAs were first detected in plasma in 2008, much more research is focusing on circulating miRNAs as biomarkers of diseases (129). MiRNAs are

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 (74). However, the mechanisms of how miRNAs are released or taken up by cells are still not clear. In addition, miRNAs have been found to be extremely stable in body fluids such as blood, but how miRNAs are protected from breakdown in the blood remains unclear. In fact, most studies in miRNAs were conducted in various samples, such as tissue, serum, human stem cells, making it difficult to compare findings across these studies.

Although circulating miR-133a was expressed in only 14 samples in this study, the relative expression level of miR-133a was positively associated with total body BMC, which contradicts previous studies (20, 134). Wang et al. (134) measured miRNAs in circulating monocytes (a type of white blood cells) in postmenopausal Caucasian women aged 57 to 68 years, and found that circulating miR-133a was highly upregulated in low BMD patients (n=10) compared to high BMD patients (n=10). Therefore, they concluded that miR-133a might be a monocyte-specific marker for osteoporosis. Chen et al. (73) conducted miRNA microarrays in isolated CD14+ (cluster of differentiation 14, known as CD14, a human gene) peripheral blood mononuclear cells (PBMCs) in Chinese women aged 50 to 59 years. They found that miR-503 was significantly downregulated in CD14+PBMCs in osteoporotic patients (n=10) compared to the healthy controls (n=10). Furthermore, CD14+PBMC cultures and mice experiments confirmed that miR-503 directly targets the RANK gene, which regulates osteoclastogenesis. Similarly, they found that miR-133a was significantly upregulated in CD14+PBMCs of osteoporotic patients.

miRNAs and Sarcopenia

In our study, we found that the relative expression level of miR-125b 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 (8, 43). Banzet et al. (8) reported that the levels of circulating miR-1, -133a, -133b and -208b were significantly increased 6 hours 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. (43) measured c-miRNA levels (miR-1, -133a, and -206) in recreational runners (n=5) before and after half marathon, and reported 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.

Muscle-specific miRNAs, such as miR-1, -206, -208, -133a/-133b, regulate many signaling pathways that are involved in muscle metabolism. For example, miR-23a, not only inhibits osteoblastogenesis in regulating bone metabolism, but also inhibits the expression of muscle atrophy genes: Muscle RING Finger I (MuRF1) and Muscle Atrophy F-box (MAFbx) (132). MuRF1 and MAFbx are upregulated in muscle atrophy (15), therefore the inhibition of MuRF1 and MAFbx by miR-23a results in the inhibition of muscle atrophy. On the other hand, miR-21 and -206 inhibit the expression

of eIF4E3 (eukaryotic translation initiation factor 4E family member 3) and PDCD10 (programmed cell death protein 10), which were both decreased during denervation-induced muscle atrophy. Therefore, the overexpression of miR-21 and -206 causes exacerbated muscle atrophy after denervation (123).

Most of the studies examining muscle-specific miRNAs have been conducted at tissue level, and there are different characteristics between tissue-specific miRNAs and circulating miRNAs (50), making it difficult to compare findings across studies. Rivas et al. (107) compared the miRNA responses to an acute bout of resistance exercise between younger and older men. Muscle biopsies were obtained 6 hours after 3 sets of 10 repetitions of knee extension and leg press exercise at 80% 1RM. MiRNAs from the skeletal muscle were analyzed using microarrays. They found that resistance exercise induced changes in miRNA expression were only observed in the younger group, suggesting impaired miRNA regulation in the older adults. They also reported that the overall miRNA score was significantly negatively associated with lower leg lean mass at rest combining younger and older participants for their analyses.

Limitations

There are several limitations to this study. First, only a small number of samples of circulating miR-1 (n=23), -133a (n=14), miR-100 (n=24) and none of circulating miR-206 were expressed. MiR-1, -133a, 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. Third, only 8 targeted miRNAs that are associated with muscle and

bone based on previous literature were included in this study due to the feasibility of the cost. Other potential miRNAs that may also be associated with osteoporosis and sarcopenia were not studied.

There are challenges in studying miRNAs as biomarkers. First, no reference values of miRNA levels have been established in healthy populations. Second, a better method needs to be developed to assess RNA purity. We used the spectrophotometer to assess the RNA purity. However, real-time PCR may be a better method to check RNA purity since serum RNA concentration is extremely low. In fact, the concentrations of 9 samples in our study were below the limit of quantification of the spectrophotometer although most of them were expressed in miRNA assays. Third, 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. Thus, it is difficult to compare results across studies between different labs and even between studies in the same lab. Fourth, the inter-individual variations in levels of circulating miRNAs are not clear. For example, physical activity, diet and presumably circadian rhythm have been reported to affect the levels of specific c-miRNAs but the exact influences need to be further studied (50). Fifth, there are over 2500 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. Last, the current miRNA tests are not cost-effective, which limits the study of miRNAs as clinical biomarkers.

Chapter 5 Conclusions

The primary purpose of the current study was to identify specific circulating miRNAs (c-miRNAs) that are associated with bone and muscle status in postmenopausal women, and to evaluate the use of these c-miRNAs as biomarkers of osteoporosis and sarcopenia. Additional purposes of this study were to determine the associations between specific c-miRNAs and bone turnover markers as well as sarcopenia parameters.

Research Questions

1. Are there significant differences (upregulation/downregulation) in bone-specific c-miRNAs (miR-21, -23a, -24, -100, and -125b) in postmenopausal women with and without osteoporosis?

   No, there were no significant differences in circulating miR-21, -23a, -24, -100, and -125b in postmenopausal women with and without osteoporosis. However, miR-21 (FC=2.59), miR-23a (FC=2.09) suggested upregulation in the osteoporosis group, whereas miR-125b (FC=0.46) suggested downregulation in the osteoporosis group.

2. What are the relationships between these bone-specific miRNAs and bone mass, bone strength?

   The relative expression level of miR-21 was significantly negatively correlated to left trochanter BMC ($r = -0.252$, $p = 0.048$), right trochanter BMC ($r = -0.294$, $p = 0.02$), and cortical vBMD at 38% of the tibia ($r = -0.253$, $p = 0.047$). There was a trend for significant association between miR-21 level and lumbar spine aBMD ($r = -0.249$, $p = 0.051$). However, no significant differences were found between the

expression levels of other bone-specific miRNAs (miR-23a, -24, -100, and -125b) and bone variables.

3. Are there significant differences (upregulation/downregulation) in skeletal muscle-specific c-miRNAs (miR-1, -133a, and -206) in postmenopausal women with and without sarcopenia?

   Because circulating miR-1, -133a, and -206 were not expressed well in this group of postmenopausal women, we were not able to answer this question.

4. What are the relationships between these muscle-specific c-miRNAs and muscle mass, muscle strength, and muscle power?

   According to the available samples that expressed muscle-specific miRNAs, the relative expression levels of circulating miR-1 and -133a were not related to muscle mass, muscle strength and muscle power.

Sub Questions

1. Are bone turnover markers, CTX, TRAP5b and PINP, associated with bone-specific miRNAs?

   Yes. The relative expression levels of miR-23a were significantly and positively correlated to the levels of bone resorption marker, TRAP5b ($r = 0.259$, $p = 0.044$). No relationships were found between miRNAs and CTX. In addition, P1NP results were inconsistent thus were not used in data analysis.

Clinical Significance

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 some bone variables, and circulating miR-125b was

significantly negatively 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.

Suggestions for Future Research

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 (41). 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 (8).

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Appendix A: IRB Approval Letter

The UNIVERSITY of OKLAHOMA

Institutional Review Board for the Protection of Human Subjects

Initial Submission – Board Approval

Date: July 18, 2016

To: Debra A Bemben, PHD

IRB#: 6971
Meeting Date: 06/27/2016
Approval Date: 07/15/2016
Expiration Date: 05/31/2017

Study Title: Bone and Muscle-specific Circulating MicroRNAs in Postmenopausal Women Based on Osteoporosis and Sarcopenia Status

Reference Number: 653487

Study Status: Active - Open

Collection/Use of PHI: Yes

At its regularly scheduled meeting the IRB reviewed the above-referenced research study. Study documents (e.g. protocol, consent, survey, etc.) associated with this submission are listed on page 3 of this letter. To review and/or access the submission forms (e.g. application) as well as the study documents approved for this submission, open this study from the *My Studies* option, click to open this study, look under Protocol Items to click on the current *Application*, *Informed Consent* and *Other Study Documents*.

If this study required routing through the Office of Research Administration (ORA), you may *not begin your study yet*, as per OUHSC Institutional policy, until the contract through ORA is finalized and signed.

As principal investigator of this research study, it is your responsibility to:

- Conduct the research study in a manner consistent with the requirements of the IRB and federal regulations at 45 CFR 46 and/or 21 CFR 50 and 56.
- Request approval from the IRB prior to implementing any/all modifications.
- Promptly report to the IRB any harm experienced by a participant that is both unanticipated and related per IRB Policy.
- Maintain accurate and complete study records for evaluation by the HRPP quality improvement program and if applicable, inspection by regulatory agencies and/or the study sponsor.
- Promptly submit continuing review documents to the IRB upon notification approximately 60 days prior to the expiration date indicated above.

In addition, it is your responsibility to obtain informed consent and research privacy authorization using the currently approved, stamped forms and retain all original, signed forms, if applicable.

1105 N. Stonewall Avenue, Oklahoma City, OK 73117 (FWA0007961)

If you have questions about this notification or using iRIS, contact the IRB @ 405-271-2045 or irb@ouhsc.edu.

Sincerely,

Vicki Lampley, MD, MPH
Vice Chairperson, Institutional Review Board

Appendix B: Informed Consent Form and HIPPA Form

Consent Form
University of Oklahoma Health Sciences Center (OUHSC)
University of Oklahoma – Norman Campus

Bone and Muscle-specific Circulating MicroRNAs in Postmenopausal Women Based on Osteoporosis and Sarcopenia Status

Principal Investigator: Debra Bemben, PhD
University of Oklahoma
405-325-2709

This is a research study. Research studies involve only individuals who choose to participate. Please take your time to make your decision. Discuss this with your family and friends.

Why Have I Been Asked To Participate In This Study?
You are being asked to take part in this study because you are a postmenopausal woman, in the age range of 70-85 years old.

Why Is This Study Being Done?
The main purpose of this study is to identify markers in the blood that are associated with decreases in bone density and muscle mass related to aging. Specifically, small substances circulating in the blood that indicate bone formation and breakdown and that control genetic processes, will be measured to evaluate their roles as indicators of bone and muscle diseases.

How Many People Will Take Part In The Study?
About 50 women will take part in this study.

What Is Involved In The Study?
If you take part in this study, three visits will be needed. However, in order to assess the reliability of our measures, a subset of about 15 participants, who would be willing to return for an additional visit, will return for a fourth visit to repeat the measurements of bone scans by two different x-ray machines (dual energy x-ray absorptiometry (DXA), peripheral quantitative computed tomography (pQCT)), functional performance and balance tests. Both types of bone scans measure bone mineral density and involve radiation exposure, however, pQCT provides additional information about bone strength. The fourth visit will be 3-7 days after the third visit.

The first visit consists of consent, blood pressure, and questionnaires, which will take approximately 40 minutes.
• Informed consent and Health Information Privacy form (HIPAA) – you must sign and date an informed consent form (this document) and HIPAA form stating that you understand all procedures and your rights as a participant.

• Health Status Questionnaire and menstrual history questionnaire– you may be excluded from the study if any answer on these questionnaires indicates you may not be eligible for this study.
• Blood pressure measurement – you will be measured at least twice to determine if you are eligible for this study or not based on blood pressure.
• Medical clearance form- if you meet all the inclusion and exclusion criteria you will be given this form to bring to your personal physician for approval to participate in this study.

The second visit consists of a venipuncture blood draw and series of tests to evaluate bone density and body composition, which will take about 3 hours. Following the blood draw, you will go to the Bone Density Laboratory, where a light breakfast will be provided, such as energy bars and orange juice, and you will be allowed to rest for about 15 minutes. Then the following measurements will be performed.

• Venipuncture blood draw (about 7 ml) (~1 hr) – will be performed by a registered nurse or phlebotomist at the OU Goddard Student Health Center in the early morning after an overnight fast to measure serum microRNAs and bone markers.
• Height and Weight (~5 min) – your height and weight will be measured.
• Series of bone scans using DXA (~40 min) – will be used to measure the bone density of your whole body, lower back and both hips and your body fat and lean tissue values. Also, your 10 year risk (FRAX score) for having a fracture related to osteoporosis will be determined. These tests are non-invasive. You will be lying on your back on the DXA table for the scans and you will be required to remain still during the procedures. DXA is a radiation procedure and is for research purposes only. There are risks associated with DXA which will be addressed below.
• Series of bone scans using pQCT (~20 min) – 3 scans on your non-dominant (non-kicking) lower leg. These tests only require you sit still in a chair while the scanner measures your lower leg at three locations. The pQCT utilizes radiation and is for research purposes only.
• Questionnaires (~35 min) – you will be given 4 health-related questionnaires to provide information about your calcium intake, physical activity levels, risk for falls, and quality of life.
• Familiarization (~15 min) – we will instruct you on correct techniques for the handgrip, gait speed, jump test and balance test. After the instruction, you will be supervised while practicing these tests at light intensities to become accustomed to the movements.

The third visit consists of the following functional performance and balance tests, which will take about a total of 45 minutes.

• Handgrip Test (~15 min) – will be measured three times for both hands with two different types of handgrip devices. You will sit on a chair with back and measured forearm supported. You will be encouraged to squeeze as hard as possible and maximum grip strength for each hand in each device will be recorded.
• Gait Speed Test (~10 min) – your walking will be assessed three times through an 8-meter straight path. The path consists of 2-meter for acceleration, 4-meter for steady-state

walking, and 2-meter for deceleration. You can use any usual walking aids, such as cane or walker. Each trial of your walking time will be recorded.
• Jump Test (~10 min) – your ability to jump will be measured three times on a jump mat with power and speed analyzer. You will be asked to do a countermovement vertical jump by crouching, then jumping with non-restricted arm motion, and then landing on the jump mat. Trained spotters will be standing on your side to help with balance, if needed. A transfer belt will be fastened around your waist to be held by the spotter to stabilize you if you lose your balance.
• Balance - Sensory Organization Test (SOT) (~10 min) – your balance will be measured by the Sensory Organization Test in NeuroCom SMART Balance Master. You will wear a vest that is attached to the harness system while standing on the platform. This harness ensures that you will not be able to fall even if your balance is disrupted. A trained spotter will stand behind you to supervise the test and protect you from falling.

The fourth visit will repeat the measurements of DXA, pQCT, functional performance and balance tests, if you would be willing to return. This will be 3-7 days after the third visit and will take about 2 hours.

How Long Will I Be In The Study?

We think that you will be in the study for 3 visits (or possibly 4 visits) lasting for a total of 4.5 hours (or possibly 6.5 hours).

There may be unanticipated circumstances under which your participation may be terminated by the investigator without regard to your consent. You may be terminated based on:

- Hypertension
- Medications impacting bone health
- Presence of metal implants at hip or spine
- Recent fractures

You can stop participating in this study at any time. However, if you decide to stop participating in the study, we encourage you to talk to the researcher first.

What Are The Risks of The Study?

While in the study, you are at risk for these side effects. However, there may also be unforeseeable risks with participation. You should discuss these with the researcher prior to providing your consent.

Risks and side effects related to having pQCT and DXA scans:
This study involves radiation exposure from 6 DXA scans and 3 pQCT scans, which are types of x-ray procedures. These procedures are for research only and not needed for your medical care. The amount of additional radiation to which you will be exposed is approximately 1% of the amount of radiation to which we are exposed annually from background sources such as the Earth and Sun. In addition to any radiographic

procedures that are being done as part of this research, you may also be exposed to radiation from procedures that are part of your normal care. The risk from radiation exposure increases over your lifetime as you receive additional exposure to radiation.

Risks and side effects related to functional performance tests:
There is slight possibility of mild soreness due to muscle strength and power testing. Additionally, there is a slight risk of injury/fall during jump or balance tests.

Are There Benefits to Taking Part in The Study?
There are no direct benefits from participating in this study.

What Other Options Are There?
There are no alternative procedures for this investigation; your alternative is to not participate.

What About Confidentiality?

Efforts will be made to keep your personal information confidential. You will not be identifiable by name or description in any reports or publications about this study. We cannot guarantee absolute confidentiality. Your personal information may be disclosed if required by law. You will be asked to sign a separate authorization form for use or sharing of your protected health information.

There are organizations that may inspect and/or copy your research records for quality assurance and data analysis. These organizations include the faculty members and graduate students appointed to this protocol from the Department of Health & Exercise Science at the University of Oklahoma, and the OUHSC Institutional Review Board.

What Are the Costs?
There is no cost to you for participating in this study.

Will I Be Paid For Participating in This Study?
There will be no compensation for participation in this study.

What if I am Injured or Become Ill While Participating in this Study?
In the case of injury or illness resulting from this study, emergency medical treatment is available. However, you or your insurance company may be expected to pay the usual charge for this treatment. No funds have been set aside by The University of Oklahoma Norman campus, to compensate you in the event of injury.

What Are My Rights As a Participant?
Taking part in this study is voluntary. You may choose not to participate. Refusal to participate will involve no penalty or loss of benefits to which you are otherwise entitled. If you agree to participate and then decide against it, you can withdraw for any reason and leave the study at any time. However, please be sure to discuss leaving the study with

the principal investigator. You may discontinue your participation at any time without penalty or loss of benefits, to which you are otherwise entitled.

We will provide you with any significant new findings developed during the course of the research that may affect your health, welfare or willingness to continue your participation in this study.

You have the right to access the medical information that has been collected about you as a part of this research study. However, you may not have access to this medical information until the entire research study has completely finished and you consent to this temporary restriction.

Whom Do I Call If I have Questions or Problems?
If you have questions, concerns, or complaints about the study or have a research-related injury, contact Dr. Debra Bemben **24/7** at 405-306-3194 or dbemben@ou.edu. If you cannot reach the Investigator or wish to speak to someone other than the investigator, contact the OUHSC Director, Office of Human Research Participant Protection at 405-271-2045.
For questions about your rights as a research participant, contact the OUHSC Director, Office of Human Research Participant Protection at 405-271-2045.

Future Communications
The researcher would like to contact you again to recruit you into future studies or to gather additional information.

_____ I give my permission for the researcher to contact me in the future.

_____ I do not wish to be contacted by the researcher again.

Signature:

By signing this form, you are agreeing to participate in this research study under the conditions described. You have not given up any of your legal rights or released any individual or entity from liability for negligence. You have been given an opportunity to ask questions. You will be given a copy of this consent document.

I agree to participate in this study:

_____	_____	_____
PARTICIPANT SIGNATURE (age ≥18) *(Or Legally Authorized Representative)*	Printed Name	Date
_____	_____	_____
SIGNATURE OF PERSON OBTAINING CONSENT	Printed Name	Date

AUTHORIZATION TO USE or SHARE
HEALTH INFORMATION[1] THAT IDENTIFIES YOU FOR RESEARCH
An Informed Consent Document for Research Participation may also be required.
Form 2 must be used for research involving psychotherapy notes.

Title of Research Project: **Bone and Muscle-specific Circulating MicroRNAs in Postmenopausal Women Based on Osteoporosis and Sarcopenia Status**

Leader of Research Team: **Dr. Debra A Bemben, PhD**

Address: **1401 Asp Avenue, Norman, OK, 73019**

Phone Number: **405-325-2709**

If you decide to sign this document, University of Oklahoma Health Sciences Center (OUHSC) researchers may use or share information that identifies you (protected health information) for their research. Protected health information will be called PHI in this document.

PHI To Be Used or Shared. Federal law requires that researchers get your permission (authorization) to use or share your PHI. If you give permission, the researchers may use or share with the people identified in this Authorization any PHI related to this research from your medical records and from any test results. Information used or shared may include all information relating to any tests, procedures, surveys, or interviews as outlined in the consent form; medical records and charts; name, address, telephone number, date of birth, race, government-issued identification numbers, and information from bone scans, functional performance and blance tests, microRNAs and bone markers.

Purposes for Using or Sharing PHI. If you give permission, the researchers may use your PHI to identify specific circulating miRNAs that are associated with bone loss and muscle loss in postmenopausal women and to evaluate the use of these circulating miRNAs as biomarkers of osteoporosis and sarcopenia.

Other Use and Sharing of PHI. If you give permission, the researchers may also use your PHI to develop new procedures or commercial products. They may share your PHI with other researchers, the research sponsor and its agents, the OUHSC Institutional Review Board, auditors and inspectors who check the research, and government agencies such as the Food and Drug Administration (FDA) and the Department of Health and Human Services (HHS), and when required by law. The researchers may also share your PHI with no one else.

[1] **Protected Health Information includes all identifiable information relating to any aspect of an individual's health whether past, present or future, created or maintained by a Covered Entity.**

Confidentiality. Although the researchers may report their findings in scientific journals or meetings, they will not identify you in their reports. The researchers will try to keep your information confidential, but confidentiality is not guaranteed. The law does not require everyone receiving the information covered by this document to keep it confidential, so they could release it to others, and federal law may no longer protect it.

YOU UNDERSTAND THAT YOUR PROTECTED HEALTH INFORMATION MAY INCLUDE INFORMATION REGARDING A COMMUNICABLE OR NONCOMMUNICABLE DISEASE.

Voluntary Choice. The choice to give OUHSC researchers permission to use or share your PHI for their research is voluntary. It is completely up to you. No one can force you to give permission. However, you must give permission for OUHSC researchers to use or share your PHI if you want to participate in the research and, if you cancel your authorization, you can no longer participate in this study.

Refusing to give permission will not affect your ability to get routine treatment or health care unrelated to this study from OUHSC.

Canceling Permission. If you give the OUHSC researchers permission to use or share your PHI, you have a right to cancel your permission whenever you want. However, canceling your permission will not apply to information that the researchers have already used, relied on, or shared or to information necessary to maintain the reliability or integrity of this research.

End of Permission. Unless you cancel it, permission for OUHSC researchers to use or share your PHI for their research will never end.

Contacting OUHSC: You may find out if your PHI has been shared, get a copy of your PHI, or cancel your permission at any time by writing to:

Privacy Official
University of Oklahoma Health Sciences Center
PO Box 26901
Oklahoma City, OK 73190

or

Privacy Board
University of Oklahoma Health Sciences Center
PO Box 26901
Oklahoma City, OK 73190

If you have questions, call: (405) 271-2511 or (405) 271-2045.

Access to Information. You have the right to access the medical information that has been collected about you as a part of this research study. However, you may not have access to this medical information until the entire research study is completely finished. You consent to this temporary restriction.

Giving Permission. By signing this form, you give OUHSC and OUHSC's researchers led by the Research Team Leader permission to share your PHI for the research project listed at the top of this form.

Patient/Participant Name (Print): _____

_____ _____
Signature of Patient-Participant Date
or Parent if Participant is a minor

Or

_____ _____
Signature of Legal Representative** Date

**If signed by a Legal Representative of the Patient-Participant, provide a description of the relationship to the Patient-Participant and the authority to act as Legal Representative:

_____
OUHSC may ask you to produce evidence of your relationship.

A signed copy of this form must be given to the Patient-Participant or the Legal Representative at the time this signed form is provided to the researcher or his representative.

Appendix C: Recruitment Materials

Female Participants Needed

Bone and Muscle-specific Circulating MicroRNAs in Postmenopausal Women Based on Osteoporosis and Sarcopenia Status

To Participate

- Women 70-85 years old
- Not current smokers
- No diabetes or uncontrolled hypertension
- No fractures within 12 months
- No metal implants at hip or spine
- Not taking medications known to affect bone or muscle mass, i.e. corticosteroids, etc. (except for osteoporosis treatment or hormone replacement)

Required Testing

- One blood draw at OU Goddard
- 6 DXA & 3 pQCT bone scans
- Functional performance tests: handgrip, gait speed and jump test
- Balance test

There are possible risks involved with participation, including risks associated with radiation exposure and blood draw.

3-4 visits required
Total time commitment about 4.5-6.5hours
Tests will take place at Huston Huffman Center Bone Density Lab, University of Oklahoma Norman Campus

If you are eligible and interested, please contact:
Zhaojing Chen at 405-837-9518, echo@ou.edu
Department of Health and Exercise Science

The University of Oklahoma is an equal opportunity institution.

Name: Zhaojing Chen
Phone: 405-837-9518
Email: echo@ou.edu

Verbal Recruitment Script

Hello, my name is Zhaojing Chen, and I am a doctoral student in the Department of Health and Exercise Science at the University of Oklahoma. I invite you to participate in a research study entitled "Bone and Muscle-specific Circulating MicroRNAs in Postmenopausal Women Based on Osteoporosis and Sarcopenia Status".

We are looking for postmenopausal women aged 70-85 years old. Potential participants must be non-smokers without diabetes, uncontrolled hypertension, or metal implants in the hip or spine. Additionally, potential participants must not have restrictions to perform functional performance or balance tests (e.g. myocardial infarction, congestive heart failure, strokes, back surgery), recent fractures within the past 6 months or taking medications known to affect bone mass or muscle mass (e.g. corticosteroids) except for osteoporosis treatment and hormone replacement drugs.

This study will include 3-4 visits to the Bone Density Lab at the University of Oklahoma. The first visit will take about 40 minutes. You will be asked to fill out the consent, health status and menstrual history questionnaire, and your blood pressure will be measured. If you are qualified, we will give you a medical clearance form to bring to your personal physician for approval to participate in this study. The second visit will last about 3 hours. One venipuncture blood draws will be performed by a registered nurse in OU Goddard in the early morning after overnight fasting. A light breakfast will be provided following blood draw. Then you will have 6 DXA scans (total body, lumbar spine, dual hip, and dual forearm) and 3 pQCT scans (3 lower leg) to assess bone health. Following that, you will be asked to fill out a series of questionnaires regarding your calcium intake, bone-specific physical activity, fall efficacy, and quality of life (SF-36). In addition, you will be instructed to practice the functional performance and balance tests. The third visit will take about 45 minutes to assess your strength, power, and balance. Handgrip strength, gait speed, and jump tests will be performed for 3 times each. Balance will be assessed using the sensory organization test on the NeuroCom SMART balance master. If you would like to return for an additional visit (15 participants needed), the measurements of DXA, pQCT, functional performance and balance tests will be repeated during the fourth visit, which will be 3-7 days after the third visit and take about 2 hours. Therefore, the total time commitment for this study will be about 4.5-6.5 hours.

There are possible risks involved with participation, including risks associated with radiation exposure, strength, power and balance tests. You will receive radiation exposure of less than 0.2 mrem from each DXA scan and less than 0.01 mrem from each pQCT scan for a total dose of 0.959 mrem, which is about the radiation received in 1 day from natural background radiation (~ 300 mrem/yr), such as naturally occurring radioactivity in soil. Although the amount of radiation exposure you will receive in this study is minimal, it is important for you to be aware that the risk from radiation exposure is cumulative over your lifetime. There is a slight possibility of mild soreness because of the muscle strength and power testings. There is a slight risk of injury/fall during walking, jumping or balance tests. Information regarding your results will be provided at the end of the study upon your request.

I would be happy to answer any additional questions that you may have about the study. Thank you!

IRB NUMBER: 6971
IRB APPROVAL DATE: 10/02/2016

Mass E-mail Script

We are looking for postmenopausal women aged 70-85 years old. Potential participants must be non-smokers without diabetes, uncontrolled hypertension, or metal implants in the hip or spine. Additionally, potential participants must not have restrictions to perform functional performance or balance tests (e.g. myocardial infarction, congestive heart failure, strokes, back surgery), recent fractures within the past 6 months or taking medications known to affect bone mass or muscle mass (e.g. corticosteroids) except for osteoporosis treatment and hormone replacement drugs.

This study will include 3-4 visits to the Bone Density Lab at the University of Oklahoma. The first visit will take about 40 minutes. You will be asked to fill out the consent, health status and menstrual history questionnaire, and your blood pressure will be measured. If you are qualified, we will give you a medical clearance form to bring to your personal physician for approval to participate in this study. The second visit will last about 3 hours. One venipuncture blood draws will be performed by a registered nurse in OU Goddard in the early morning after overnight fasting. A light breakfast will be provided following blood draw. Then you will have 6 DXA scans (total body, lumbar spine, dual hip, and dual forearm) and 3 pQCT scans (3 lower leg) to assess bone health. Following that, you will be asked to fill out a series of questionnaires regarding your calcium intake, bone-specific physical activity, fall efficacy, and quality of life (SF-36). In addition, you will be instructed to practice the functional performance and balance tests. The third visit will take about 45 minutes to assess your strength, power, and balance. Handgrip strength, gait speed, and jump tests will be performed for 3 times each. Balance will be assessed using the sensory organization test on the NeuroCom SMART balance master. If you would like to return for an additional visit (15 participants needed), the measurements of DXA, pQCT, functional performance and balance tests will be repeated during the fourth visit, which will be 3-7 days after the third visit and take about 2 hours. Therefore, the total time commitment for this study will be about 4.5-6.5 hours. There are possible risks involved with participation, including risks associated with radiation exposure, strength, power and balance tests.

If you are interested in this study and for more information, please contact Zhaojing Chen via email at echo@ou.edu.

The OU IRB has approved the content of this message but not the method of distribution. The OU IRB has no authority to approve distribution by mass email.

IRB NUMBER: 6971
IRB APPROVAL DATE: 10/02/2016

Appendix D: Questionnaires

Participant Medical Clearance Form

University of Oklahoma Bone Density Laboratory

Dear Doctor,

Your patient, _____, has indicated that she wishes to participate in a research study investigating the specific circulating microRNAs that are associated with bone loss and muscle loss in 70-85 years old postmenopausal women. This study involves 3-4 testing sessions: (1) completion of an informed consent form, health status and menstrual history questionnaires, and blood pressure measurements; (2) completion of venipuncture blood draws by a registered nurse or phlebotomist in OU Goddard in the early morning after overnight fasting, 6 DXA (total body, lumbar spine, dual hip, and dual forearm) and 3 pQCT bone scans (lower leg), questionnaires of calcium intake, bone-specific physical activity, fall efficacy scale, and quality of life (SF-36), and familiarization with handgrip, gait speed, jump and balance test procedures; (3) completion of a handgrip strength test for 3 times for each hand using 2 types of devices, gait speed testing involving 3 trials of walking on an 8-meter path with usual speed, jump testing involving a total of 3 maximal vertical jumps on jump mat; and possibly (4) repeat the measurements of DXA, pQCT, functional performance and balance tests if she is willing to return. Proper safety precautions will be taken during the entire protocol. Prior to participation, participant is required to obtain medical clearance from her personal physician(s). Specific inclusion and exclusion criteria apply to the participants recruited for this study. Below are the exclusion criteria.

- Current smokers;
- Having diabetes;
- Having uncontrolled hypertension;
- Recent fractures (during the preceding 12 months);
- Having restrictions to perform handgrip strength, gait speed, jump or balance tests, e.g. myocardial infarction/congestive heart failure/strokes within the past 6 months;
- Degenerative neuromuscular conditions, e.g. Parkinson's disease;
- Taking medications known to affect bone mass or muscle mass, e.g. corticosteroids, selective serotonin reuptake inhibitors (except for osteoporosis treatment or hormone replacement drugs);
- Joint replacement or metal implants at hip or spine

☐ I recommend that the above-named individual be allowed to participate in the study.

☐ **I do not** recommend that the above-named individual be allowed to participate in the study.

MEDICATIONS/NOTES: _____

Physician Name: _____ Contact Number: _____
(please print)

Physician Signature: _____ Date: _____

This form can be faxed to (405) 325-0594 or emailed to echo@ou.edu. Thank you!

This study has been approved by the University of Oklahoma Institutional Review Board. *For questions, please contact Debra A. Bemben, Ph.D. at (405) 325-2709 or dbemben@ou.edu.*

IRB NUMBER: 6971
IRB APPROVAL DATE: 10/02/2016

Bone Density Research Laboratory

OU Department of Health and Exercise Science

Health Status Questionnaire

Instructions Complete each question accurately. All information provided is confidential.

(NOTE: The following codes are for office use only: RF; MC)

Part 1. Information about the individual

1._____

Date

2. _____

Legal name Nickname

3._____

Mailing address

_____

Home phone Business/cell phone

4.Gender (circle one): Female Male (RF)

5. Year of birth: _____ Age _____

6. Number of hours worked per week:

NA (retired) Less than 20 20-40 41-60 Over 60

If not retired, more than 25% of time spent on job (circle all that apply)

Sitting at desk Lifting or carrying loads Standing Walking Driving

Part 2. Medical history

7. (RF) Circle any who died of heart attack before age 50:

Father Mother Brother Sister Grandparent

8.Date of: Last medical physical exam: _____Last physical fitness test: _____

Year Year

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9. Circle operations you have had:

Back	Heart (MC)	Kidney	Eyes	Joint	Neck
Ears	Hernia	Lung	Other _____		NONE

10. Please circle any of the following for which you have been diagnosed or treated by a physician or health professional:

Alcoholism	Diabetes	Kidney problem (MC)
Anemia, sickle cell	Emphysema	Mental illness
Anemia, other	Epilepsy	Neck strain
Asthma	Eye problems	Obesity (RF)
Back strain	Gout	Osteoporosis
Bleeding trait	Hearing loss	Phlebitis (MC)
Bronchitis, chronic	Heart problems	Rheumatoid arthritis
Cancer	High blood pressure (RF)	Stroke (MC)
Cirrhosis, liver (MC)	Hypoglycemia	Thyroid problem
Concussion (MC)	Hyperlipidemia (RF)	Ulcer
Congenital defect	Infectious mononucleosis (MC)	Other _____
NONE		

11. Circle all medicine taken in last 6 months:

Asthma (list type) _____ High-blood-pressure medication (list type) _____

Blood thinner (MC)	Epilepsy medication	Thyroid
Corticosteroids	Estrogen	Other _____
Depression	Heart-rhythm medication (MC)	NONE
Diabetic pill	Insulin (MC)	
Digitalis (MC)	Nitroglycerin (MC)	
Diuretic (MC)		

12. Any of these health symptoms that occurs frequently is the basis for medical attention. Circle the number indicating how often you have each of the following:

1 = Practically never 2 = Infrequently 3 = Sometimes 4 = Fairly often 5 = Very often

a. Cough up blood (MC)
1 2 3 4 5

b. Abdominal pain (MC)
1 2 3 4 5

c. Low back pain (SLA)
1 2 3 4 5

d. Leg pain (MC)
1 2 3 4 5

e. Arm or shoulder pain (MC)
1 2 3 4 5

f. Chest pain (RF) (MC)
1 2 3 4 5

g. Swollen joints (MC)
1 2 3 4 5

h. Feel faint (MC)
1 2 3 4 5

I. Dizziness (MC)
1 2 3 4 5

j. Breathless with slight exertion (MC)
1 2 3 4 5

Part 3. Health-related behavior

13. (RF) Do you now smoke? Yes No

14. If you are a smoker, indicate number smoked per day:

Cigarettes: 40 or more 20-39 10-19 1-9
Cigars or pipes only: 5 or more or any inhaled Less than 5, none inhaled

15. Weight now: _____lb. One year ago: _____lb. Age 21: _____lb.

16. Do you regularly engage in strenuous exercise or hard physical labor?

1. Yes (answer question # 19) 2. No (stop)

17. Do you exercise or labor at least three times a week?

1. Yes 2. No

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Bone Density Research Laboratory

Department of Health and Exercise Science, University of Oklahoma

MENSTRUAL HISTORY QUESTIONNAIRE

Participant ID: _____ Date: _____

We are asking you to give us as complete a menstrual history as possible. All information you provide will be strictly confidential.

SECTION A. CURRENT MENSTRUAL STATUS

1. At what age did you experience your final menstrual period?

2. Have you had a hysterectomy (surgical removal of the uterus)? If yes, at what age did you have this surgery?

3. Have you had your ovaries removed? If yes, at what age did you have this surgery?

4. Are you currently on estrogen and/or progesterone replacement therapy? If no, skip to question 5.

If yes, how long have you been on hormone replacement therapy?

What are the brand name, dosage, and type (e. g., pills, cream, patch) of hormone medication you are taking?

5. Have you taken estrogen and/or progesterone replacement in the past? If no, skip to SECTION B.

If yes, what was the type (e.g., pills, cream, patch) and dosage of the medication?

At what age did you start taking hormone replacement?

How long did you continue taking the hormone replacement?

At what age and why did you stop taking hormone replacement?

6. If you answered yes to questions 4 or 5, did you experience any side effects (e.g., weight gain, mood swings, headaches) while taking hormone replacement? If yes, please list the side effects.

SECTION B: PAST MENSTRUAL HISTORY

1. Approximately at what age did you experience your first menstrual period?

2. Were your periods regular (occurring monthly) during the first two years after menstruation began? If no, at what age did your periods eventually become regular?

3. Did you perform any form of athletic training prior to your first menstrual period? If yes, indicate type of training (e.g., gymnastics, track, basketball, etc.) and the number of years you trained for each activity.

4. Has there been any time in the past where your periods were irregular or absent? If no, skip to question 5.

   If yes, did these periods coincide with unusual bouts of training, or with a period of stress? How long did this occur?

5. Have you ever consulted a doctor about menstrual problems (specifically, about irregular or missing periods)? If no, skip to question 6.

   If yes, what was the diagnosis (e.g., shortened luteal phase, amenorrhea)?

   Have you ever been tested to determine if you were ovulating normally?

6. Have you ever consulted a physician about any problems relating to your hormonal system? If so, please explain.

BONE DENSITY RESEARCH LABORATORY
DEPARTMENT OF HEALTH AND EXERCISE SCIENCE
UNIVERSITY OF OKLAHOMA

CALCIUM INTAKE ESTIMATION

NAME:_____ _____ TODAY'S DATE:___ _____

Complete this form (where indicated) to represent your dietary intake in the past year.

Tally *(office use only)*	Score *(office use only)*	Food Type	serving size	EAT THIS FOOD: EVERY WEEK write in # servings/week	EVERY DAY write in # servings/day
	300	Milk- whole, 2% skim	1 cup		
	150	Cheese food or spread	1 oz		
	150	Cheese sauce	1/4 cup		
	150	American cheese	1 slice		
	150	Cottage cheese	1 cup		
	350	Ricotta cheese	1 cup		
	150	Blue cheese	¼ cup		
	200	Natural cheese (except cream cheese) includes cheddar, Swiss, mozzarella, and so forth	1 oz		
	285	Buttermilk	1 cup		
	300	Yogurt, flavored or plain	1 cup		
	450	Fast Food Milkshake	12 oz		
	165	Cocoa from mix	1 packet		
	330	Eggnog	1 cup		
	280	Chocolate milk	1 cup		
	250	Macaroni and cheese, cheese soufflé, lasagna, quiche, carmelloni, pizza	1 serving		
	180	Cream corn or chowder with milk	1 cup		
	115	Almonds	1/3 cup		
	180	Broccoli	1 cup		
	85	Beet greens, spinach	½ cup		
	160	Baked beans	1 cup		
	100	Figs	5 dried		
	140	Scalloped potatoes	1 cup		
	150	Soybeans	1 cup		
	150	Tofu	½ cup		

PLEASE TURN OVER

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Tally (office use only)	Score (office use only)	Food Type	serving size	write in # servings/week	write in # servings/day
	30	Bread, white or whole grain	1 slice		
	120	Waffle or pancake	1 large		
	50	Muffin, biscuit, cornbread	1 medium		
	40	Rolls, buns	½		
	225	Egg McMuffin	1		
	130	Fast food cheeseburger or hamburger	1		
	310	Enchilada or bean burrito	1		
	125	Creamed fish and meats	1 cup		
	170	Shellfish, cooked	4 oz		
	200	Canned salmon with bones	½ cup		
	200	Sardines, smelts, herring	½ cup		
	100	Fudgesicle	1		
	125	Custard pie	1 slice		
	175	Ice cream or ice milk	1 cup		
	190	Pudding with milk	½ cup		
	203	Frozen yogurt	1 cup		

Please list any dietary supplements (single and multi-vitamins, calcium, herbal etc.) you take below, including the brand name and amount (mg)

1. _____ _____
2. _____ _____
3. _____ _____ _____
4. _____ _____
5. _____ _____ _____

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IRB APPROVAL DATE: 07/15/2016

Bone-Specific Physical Activity Questionnaire (BPAQ)

SUBJECT ID:	DATE:

1. Please list <u>any</u> sports or other physical activities you have participated in regularly. Please <u>tick</u> the boxes to indicate how old you were for each sport/activity and how many years you participated for.

Age: Activities	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25

Age: Activities	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50

Age: Activities	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75

Age: Activities	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100

Bone-Specific Physical Activity Questionnaire (BPAQ)

SUBJECT ID:	DATE:

2. Please list the sports or other physical activities (be as specific as possible) you participated in regularly during the last 12 months and indicate the average frequency (sessions per week)?

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

BONE-SPECIFIC PHYSICAL ACTIVITY QUESTIONNAIRE
Developed by B.K. Weeks and B.R. Beck
Griffith University, QLD, Australia

Appendix E: Serum CrossLaps (CTX-1) ELISA Kit Instruction

immunodiagnosticsystems

Serum CrossLaps®
(CTX-I) ELISA

For the quantification of degradation products of C-terminal telopeptides of Type I collagen in human serum and plasma

CE REF AC-02F1 Σ 96

1

INTRODUCTION

Intended use

The Serum CrossLaps® (CTX-I) ELISA is an enzyme immunological test for the quantification of degradation products of C-terminal telopeptides of Type I collagen in human serum and plasma.

The Serum CrossLaps® (CTX-I) ELISA assay is intended for in vitro diagnostic use as an indication of human bone resorption and may be used as an aid in

A. Monitoring bone resorption changes of

1) Anti resorptive therapies in postmenopausal women:
a) Hormone Replacement Therapies (HRT) with hormones and hormone like drugs
b) Bisphosphonate therapies

2) Anti resorptive therapies in individuals diagnosed with osteopenia;
a) Hormone Replacement Therapies (HRT) with hormones and hormone like drugs
b) Bisphosphonate therapies

B. Predicting skeletal Response (Bone Mineral Density) in postmenopausal women undergoing anti resorptive therapies

a) Hormone Replacement Therapies (HRT) with hormones and hormone like drugs
b) Bisphosphonate therapies

Limitations

The use of the test has not been established to predict the development of osteoporosis or future fracture risk.
The use of the test has not been established in hyperparathyroidism or hyperthyroidism.
When using the test to monitor therapy, results may be confounded in patients afflicted with clinical conditions known to affect bone resorption e.g. bone metastases, hyperparathyoidism or hyperthyroidism.
Serum CrossLaps® (CTX-I) ELISA results should be interpreted in conjunction with clinical findings and other diagnostic results and should not be used as a sole determinant in initiating or changing therapy
Do not interchange Serum CrossLaps® (CTX-I) ELISA values with Urine CrossLaps® (CTX-I) ELISA values.

Summary and explanation of the test

Type I collagen accounts for more than 90% of the organic matrix of bone and is synthesized primarily in bone (1). During renewal of the skeleton, Type I collagen is degraded, and small peptide fragments are excreted into the bloodstream. These fragments can be measured by Serum CrossLaps® (CTX-I) ELISA. The measurements of the specific degradation products of Type I collagen in both urine (2) and serum (3) by a competitive CrossLaps have been reported.
The sandwich assay has been reported as useful for follow up of anti resorptive treatment of patients with metabolic bone diseases (3-17).

Principle of the procedure

The Serum CrossLaps® (CTX-I) ELISA is based on two highly specific monoclonal antibodies against the amino acid sequence of EKAHD-ß-GGR, where the aspartic acid residue (D) is ß-isomerized. In order to obtain a specific signal in the Serum CrossLaps® (CTX-I) ELISA, two chains of EKAHD-ß-GGR must be cross linked. Standards, control, or unknown serum samples are pipetted into the appropriate microtitre wells coated with streptavidin, followed by application of a mixture of a biotinylated antibody and a peroxidase conjugated antibody. Then, a complex between CrossLaps antigens, biotinylated antibody and peroxidase conjugated antibody is generated, and this complex binds to the streptavidin surface via the biotinylated antibody. Following the one step incubation at room temperature, the wells are emptied and washed. A chromogenic substrate is added and the colour reaction is stopped with sulfuric acid. Finally, the absorbance is measured.

PRECAUTIONS

The following precautions should be observed in the laboratory:

- Do not eat, drink, smoke or apply cosmetics where immunodiagnostic materials are being handled
- Do not pipette by mouth.
- Wear gloves when handling immunodiagnostic materials and wash hands thoroughly afterwards
- Cover working area with disposable absorbent paper

Warnings

For in vitro use only.

- All reagents and laboratory equipment should be handled and disposed of as if they were infectious.
- Do not use kit components beyond the expiry date and do not mix reagents from different lots.

HAMA interference

Some individuals have antibodies to mouse immunoglobulins (HAMA), which can cause interference in immunoassays that employ murine monoclonal antibodies, such as Serum CrossLaps. In rare cases, the content of HAMA exceeds the capacity of the blocking agent incorporated into Serum CrossLaps leading to a false-positive test result. Therefore, Serum CrossLaps values should be used only in conjunction with information available from the clinical evaluation of the patient.

Storage

Store the Serum CrossLaps® (CTX-I) ELISA kit upon receipt at 2-8°C. Under these conditions the kit is stable up to the expiry date stated on the box.

Note:

The specimens' storage and stability information stated above are general recommendations for use in a variety of settings of laboratories. Each laboratory should follow the guidelines or requirements of local, state, and/or federal regulations or accrediting organizations to establish its own specimens handling and storage stability. For guidance on appropriate practices, please refer to the CLSI GP44-A4, Procedures for the Handling and Processing of Blood Specimens for Common Laboratory Tests; Approved Guideline - Fourth Edition

MATERIALS

Specimen collection

Collect blood by venipuncture taking care to avoid haemolysis. Separate the serum from the cells within 3 hours after collection of blood. It is recommended to freeze (<-18°C) samples immediately.

For optimal results it is recommended to draw blood as fasting morning samples (18).

Also for monitoring the individual patient, follow up samples should be collected under same conditions as the baseline sample.

When analysing plasma, both heparia and EDTA plasma may be used.

Materials supplied

Before opening the kit, read the section on Precautions. The kit contains reagents sufficient for 96 determinations.

Streptavidin coated microtitre plate MICROPLAT

Microwell strips (12x8 wells) pre-coated with streptavidin. Supplied in a plastic frame.

CrossLaps Standard CAL 0

One vial (min. 5.0 mL/vial) of ready for use PBS buffered solution with protein stabiliser and preservative.

CrossLaps Standards CAL 1 - 5

Five vials (min. 0.4 mL/vial) of ready for use, CrossLaps standard in a PBS buffered solution with protein stabiliser and preservative.The exact value of each Standard is printed on the QC Report.

Control CTRL 1 - 2

Two vials (min. 0.4 mL/vial) of ready for use, desalted urinary antigens of human origin in a PBS buffered solution with protein stabiliser and preservative. Please refer to enclosed QC Report for control range.

Biotinylated Antibody Ab BIOTIN
One vial (min. 0.25 mL) of a concentrated solution of a biotinylated monoclonal murine antibody specific for degradation products of C-terminal telopeptides of Type I collagen, raised. Prepared in a buffered solution with protein stabiliser and preservative.

Peroxidase Conjugated Antibody ENZYMCONJ
One vial (min. 0.25 mL) of a concentrated solution of a peroxidase conjugated murine monoclonal antibody specific for degradation products of C-terminal telopeptides of Type I collagen. Prepared in a buffered solution with protein stabiliser and preservative.

Incubation Buffer BUF
One vial (min. 19 mL) of a ready for use buffered solution with protein stabiliser, detergent and preservative.

Substrate Solution SUBS TMB
One vial (min. 12 mL) of a ready for use tetramethylbenzidine (TMB) substrate in an acidic buffer.
Please note that the chromogenic substrate might appear sligthy blueish.

Stopping Solution H2SO4
One vial (min. 12 mL) of ready for use 0.18 mol/L sulfuric acid.

Washing Buffer WASHBUF 50x
One vial (min. 20 mL) of a concentrated washing buffer with detergent and preservative.

Sealing tape
Adhesive film for covering wells during incubation.

Materials required — not supplied
- Containers for preparing the Antibody Solution and the Washing Solution
- Precision micropipettes to deliver 50-200 µL
- Distilled water
- Precision 8 or 12 channel multipipette to deliver 100 µL, and 150 µL
- Microwell mixing apparatus
- Microtiter plate reader

ASSAY PROCEDURE

Mix all reagents and samples before use (avoid foam)

Determine the number of strips needed for the assay. It is recommended to test all samples in duplicate. In addition, for each run a total of 16 wells are needed for the standards and controls. Place the appropriate number of strips in the plastic frame. Store unused immuno strips in the tightly closed foil bag with desiccant capsules.

Prior to use, prepare and equilibrate all solutions to room temperature. **Perform the assay at room temperature (18-22°C).**

1 **Preparation of the Antibody Solution:**
ATTENTION: Prepare the following Antibody Solution maximum 30 minutes before starting the assay. Mix the Biotinylated Antibody Ab BIOTIN, Peroxidase Conjugated Antibody ENZYMCONJ and Incubation Buffer BUF in the volumetric ratio 1+1+100 in an empty container. Mix carefully and avoid formation of foam. **Prepare a fresh solution before each run of the assay.**

2 **One Step incubation**
Pipette 50 µL of either **Standards** CAL 0 - 5, **Control** CTRL 1 - 2, or unknown samples into appropriate wells followed by 150 µL, of the **Antibody Solution**. Cover the immunostrips with sealing tape and incubate for 120±5 minutes at room temperature (18-22°C) on a microtitre plate mixing apparatus (300 rpm).

3 **Washing**
Wash the immunostrips 5 times manually with 300 µL diluted **Washing Buffer** (**WASHBUF 50x** diluted 1+50 in distilled water). Using an automated plate washer, follow the instructions of the manufacturer or the guidelines of the laboratory. Usually 5 washing cycles are adequate. Make sure that the wells are **completely emptied** after each manual or automatic washing cycle.

4 **Incubation with chromogenic substrate solution**
Pipette 100 µL of the **Substrate Solution** **SUBS TMB** into each well and incubate for 15±2 minutes at room temperature (18-22°C) in the dark on the mixing apparatus (300 rpm). Use sealing tape.
Do not pipette directly from the vial containing TMB substrate but transfer the needed volume to a clean reservoir. Remaining substrate in the reservoir should be discarded and not returned to vial TMB.

5 **Stopping of colour reaction**
Pipette 100 µL of the **Stopping Solution** **H2SO4** into each well.

6 **Measurement of absorbance**
Measure the absorbance at 450 nm with 650 nm as reference within two hours.

Limitations of the procedure
If the absorbance of a sample exceeds that of **Standard 5,** the sample should be diluted in **Standard 0** and re-analysed.

QUALITY CONTROL

Good Laboratory Practice (GLP) requires the use of quality control specimens in each series of assays in order to check the performance of the assay. Controls should be treated as unknown samples, and the results analysed with appropriate statistical methods.

RESULTS

Calculation of results
A quadratic curve fit can be used.

Alternatively, calculate the mean of the duplicate absorbance determinations. Construct a standard curve on graph paper by plotting the mean absorbances of the six standards 0-5 (ordinate) against the corresponding CrossLaps concentrations (abscissa). Determine the CrossLaps concentration of the controls and each patient sample by interpolation.

Example of results obtained:

Standards/ Controls/ Samples	CrossLaps conc. (ng/mL)	$A_{450-860}$ (nm) Obs 1/ Obs 2	Mean $A_{450-860}$ (nm)	Interpolated CrossLaps conc. (ng/mL)
Standard 0	0.000	0.066 / 0.065	0.066	
Standard 1	0.178	0.210 / 0.209	0.210	
Standard 2	0.489	0.472 / 0.448	0.460	
Standard 3	0.960	0.844 / 0.819	0.832	
Standard 4	1.902	1.598 / 1.560	1.579	
Standard 5	2.494	2.061 / 2.004	2.033	
Control 1		0.349 / 0.354 0.918 /	0.352	0.355
Control 2		0.952	0.935	1.086
Sample I		0.140 / 0.138	0.139	0.091
Sample II		0.447 / 0.439	0.443	0.469
Sample III		1.305 / 1.303	1.304	1.555

Appendix F: MicroVue TRAP5b EIA Kit Instruction

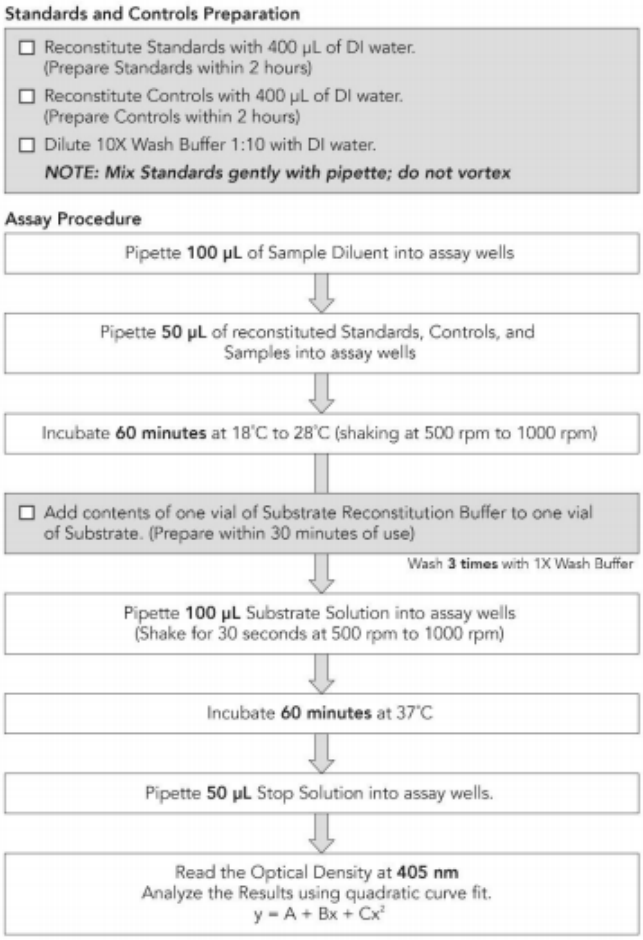
QUIDEL

MicroVue™ Bone
TRAP5b EIA

An immunocapture enzyme assay for the determination of tartrate-resistant acid phosphatase isoform 5b in human serum or plasma

For *In Vitro* Diagnostic Use. For export only. Not for sale or use in the United States or Canada.

SUMMARY



REAGENTS AND MATERIALS PROVIDED

40 Assays for TRAP5b conducted in duplicate (96 wells)

MicroVue TRAP5b Assay kit contains the following:

A | E	**TRAP5b Standards:** (lyophilized) recombinant protein composed of Human TRAP5b. The exact concentration is stated on each vial	**Parts 0711631-71**	**0.4 mL, 2 each**
L H	**Low/High Controls** (lyophilized) recombinant protein composed of Human TRAP5b. The concentration range is stated on the kit Certificate of Analysis (C of A)	**Parts 0711681-91**	**0.4 mL, 2 each**
❶	**Microwell Plate** 12 x 8 wells coated with murine monoclonal anti-TRAP5b antibodies	**Part 0711611**	**12 each**
❷	**Stop Solution** 0.2N sodium hydroxide (NaOH)	**Part 07116C1**	**12 mL**
❸	**10X Wash Buffer** TBS/Tween. Contains 0.5% Tween® 20 and 0.02% ProClin® 300	**Part 07116D1**	**100 mL**
❹	**Sample Diluent** Tris buffer. Contains 0.02% ProClin 300	**Part 0711621**	**20 mL**
❺	**Substrate Reconstitution Buffer** MES buffer. Contains 0.02% ProClin 300	**Part 07116B1**	**12 mL, 2 each**
❻	**Substrate** Substrate dissolving solution, 2-chloro-4-nitrophenyl-phosphate powder (CNPP)	**Part 07116A1**	**12 mL, 2 each**
	Plate Tape Cover	**Part 0047**	**3 each**

Tween® 20 is a registered trademark of ICI Americas Inc.
ProClin® is a registered trademark of Rohm and Haas Company.

MATERIALS REQUIRED BUT NOT PROVIDED

- Adjustable micropipettes for dispensing 50, 100, 300 µL, both single and multi-channel
- Microplate shaker capable of constant shaking at 500-1000 rpm for 60 minutes
- Incubator at 37°C
- Labware suitable for liquid measurement of 10-300 mL
- Deionized or distilled water
- Microplate reader capable of reading at 405 nm
- Computer
- Software package facilitating data generation, quadratic curve fit, and data analysis
- Suitable device for washing the microplate
- Graduated pipette or equivalent for dispensing 12 mL
- Absorbent material for blotting the in-process microplate after washing

WARNINGS AND PRECAUTION

- For *in vitro* diagnostic use
- Treat specimen samples as potentially biohazardous material. Follow Universal Precautions when handling contents of this kit and any patient samples.

Standards

Add 400 µL of deionized (distilled) water to the vial containing lyophilized Standard and dissolve for at least 5 minutes. Mix thoroughly. The reconstituted Standards should be used within 2 hours if stored at 18°C to 28°C or within 24 hours if stored at 4°C.

Controls

Add 400 µL of deionized (distilled) water to the vials containing lyophilized Controls, and dissolve for at least 5 minutes. Mix thoroughly. The reconstituted Controls should be used within 2 hours if stored at 18°C to 28°C or within 24 hours if stored at 4°C.

10X Wash Buffer

Dilute 100 mL of 10X Wash Buffer with 900 mL deionized (distilled) water. The working Wash Buffer is stable for 1 month at 18°C to 28°C.

Substrate Solution

Prepare Working Substrate Solution by adding the contents of one vial of Substrate Reconstitution Buffer to the contents of 1 vial of Substrate. Prepare within 30 minutes of use.

Stop Solution

Stop Solution is provided ready to use.

ASSAY PROCEDURE

Read entire product insert before beginning the assay.
See WARNINGS AND PRECAUTIONS and REAGENT PREPARATION.

Determine amount of each reagent required for the number of strips to be used.

# of Strips	4	6	8	12
# of Samples (tested in duplicate)	8	16	24	40
Substrate (vial)	1	1	1	1
1X Wash Buffer (mL)	100	150	200	300

Sample/Enzyme Incubation

1. Allow pouch of Coated Strips to equilibrate to 18°C to 28°C before opening. Remove Stripwell Frame and the required number of Coated Strips from the pouch. Ensure that the pouch containing any unused strips is completely resealed and contains desiccant.
2. Pipette 100 µL of Sample Diluent into microplate wells.
3. Pipette 50 µL of each reconstituted Standard, Control and sample into appropriate microplate wells.
4. Seal the microwell plate with supplied plate tape cover and incubate for 60 minutes at 18°C to 28°C on a microplate shaker set at 500 rpm to 1000 rpm.
5. After incubation, wash the microplate wells three times with a minimum of 300 µL of Wash Buffer per well. After washing, tap the wells gently on a paper towel to expel any remaining liquid.

Substrate Incubation

6. Pipette 100 µL of Working Substrate Solution into each well.
7. Seal the microplate and mix on a microplate shaker for 30 seconds at 500–1000 rpm. After shaking,m incubate for 60 minutes in a 37°C incubator.

Stop/Read

8. Pipette 50 µL of Stop Solution into each well to stop the reaction.
9. Read and record the absorbance of each well at 405 nm.
10. Use a quadratic curve fit for the standard curve. Calculate the values of Controls and specimens from the standard curve.

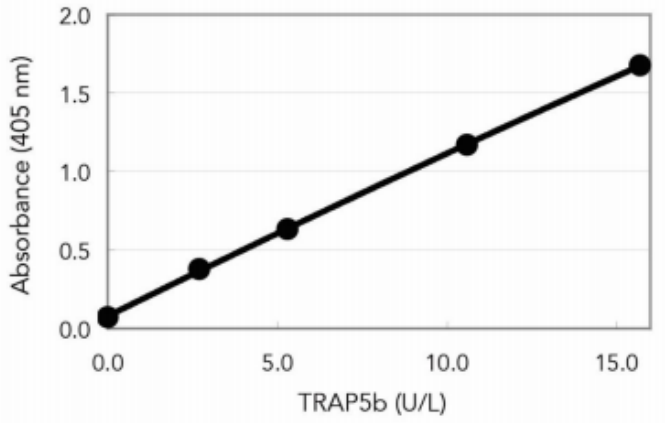
QUALITY CONTROL

The Certificate of Analysis included in this kit is lot specific and is to be used to verify that the results obtained by your laboratory are similar to those obtained at Quidel Corporation.

Quality control ranges are provided. The control values are intended to verify the validity of the curve and sample results. Each laboratory should establish its own parameters for acceptable assay limits. If the control values are NOT within your laboratory's acceptance limits, the assay results should be considered questionable, and the samples should be tested again.

INTERPRETATION OF RESULTS

Representative Standard Curve



OBSERVED VALUES

Observed serum values for TRAP5b activity in healthy men and women are reported as follows:

Gender	Age (years)	n	Mean (U/L)
Men	≥ 20	91	4.0 ± 1.4
Women (Premenopausal)	30 to 44	31	2.9 ± 1.4
Women (Postmenopausal)	≥ 50	36	4.3 ± 1.5

Observed TRAP5b values (U/L) in 64 healthy adults (see gender and age information below) using both serum and plasma (Heparin) collection methods. Plasma samples were run for comparison to serum results.

Appendix G: Qiagen miRNeasy Serum/Plasma Handbook

February 2012

miRNeasy Serum/Plasma Handbook

miRNeasy Serum/Plasma Kit
For purification of total RNA, including miRNA, from animal and human plasma and serum

miRNeasy Serum/Plasma Spike-In Control
For normalization of miRNA purification from serum or plasma

Sample & Assay Technologies

Contents

Kit Contents

miRNeasy Serum/Plasma Kit	(50)
Catalog no.	**217184**
Number of preps	**50**
RNeasy® MinElute® Spin Columns (each packaged with a 2 ml Collection Tube)	50
Collection Tubes (1.5 ml)	50
Collection Tubes (2 ml)	50
QIAzol® Lysis Reagent*	50 ml
Buffer RWT*†	15 ml
Buffer RPE‡	11 ml
Ce_miR-39_1 miScript® Primer Assay	(100)
RNase-Free Water	10 ml
Quick-Start Protocol	1

* Contains a guanidine salt. Not compatible with disinfectants containing bleach. See page 6 for safety information.

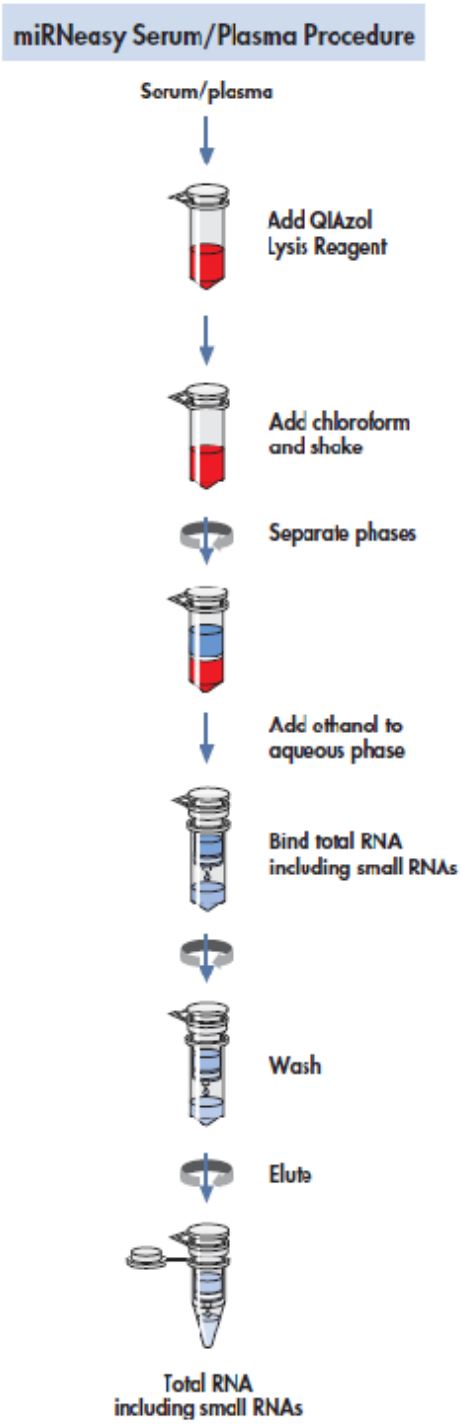
† Buffer RWT is supplied as a concentrate. Before using for the first time, add 2 volumes of ethanol (96%–100%) as indicated on the bottle to obtain a working solution.

‡ Buffer RPE is supplied as a concentrate. Before using for the first time, add 4 volumes of ethanol (96–100%) as indicated on the bottle to obtain a working solution.

miRNeasy Serum/Plasma Spike-In Control	(10 pmol)
Catalog no.	**219610**
Lyophilized *C. elegans* miR-39 miRNA mimic	10 pmol

Shipping and Storage

The miRNeasy Serum/Plasma Kit (cat. no. 217184) is shipped at ambient temperature. Store the RNeasy MinElute spin columns immediately at 2–8°C. QIAzol Lysis Reagent can be stored at room temperature (15–25°C) or at 2–8°C. Store the Ce_miR-39_1 miScript Primer Assay at –20°C either lyophilized or reconstituted (see next paragraph). Store the remaining components dry at room temperature. All kit components are stable for at least 9 months under these conditions.


miRNeasy Serum/Plasma Procedure
Serum/plasma
Add QIAzol Lysis Reagent
Add chloroform and shake
Separate phases
Add ethanol to aqueous phase
Bind total RNA including small RNAs
Wash
Elute
Total RNA including small RNAs

Protocol: Purification of Total RNA, Including miRNA, from Serum and Plasma

This protocol is intended as a guideline for the purification of cell-free total RNA, which primarily includes small RNAs such as miRNAs, from small volumes (up to 200 µl) of serum and plasma using the miRNeasy Serum/Plasma Kit. The protocol can also be used for small volumes of other body fluids such as urine. Processing of more than 200 µl sample is not recommended, because the amounts of contaminants introduced by larger sample volumes may interfere with the purification process.

For recommendations on collection, preparation, and storage of cell-free plasma and serum, see Appendix A, page 22.

This protocol requires miRNeasy Serum/Plasma Spike-In Control (cat. no. 219610), which must be ordered separately.

Important points before starting

- After collection and centrifugation, plasma or serum can be stored at 2–8°C for up to 6 hours or used directly in the procedure. For long-term storage, freezing at –20°C or –80°C in aliquots is recommended. To process frozen lysates, incubate at 37°C in a water bath until samples are completely thawed and salts are dissolved. Avoid prolonged incubation, which may compromise RNA integrity.
- DNase I digestion is not recommended for plasma or serum samples. Cell-free body fluids typically do not contain significant amounts of DNA, and the combined QIAzol and RNeasy technologies efficiently remove most of the trace amounts of DNA in plasma and serum. In addition, miScript Primer Assays and most other assays for mature miRNA are not affected by the presence of small amounts of genomic DNA. On-column DNase treatment may reduce recovery of small RNA from plasma or serum.
- Buffer RWT may form a precipitate upon storage. If necessary, redissolve by warming and then place at room temperature (15–25°C).
- QIAzol Lysis Reagent and Buffer RWT contain a guanidine salt and are therefore not compatible with disinfecting reagents containing bleach.
- Except for phase separation (step 7), all protocol and centrifugation steps should be performed at room temperature.
- The procedure is suitable for use with either serum samples or plasma samples containing citrate or EDTA. Plasma samples containing heparin should not be used because this anticoagulant can interfere with downstream assays, such as RT-PCR.

Things to do before starting

- Buffers RWT and RPE are supplied as concentrates. Before using for the first time, add the required volumes of ethanol (96–100%), as indicated on the bottle, to obtain a working solution.
- Prepare a working solution of miRNeasy Serum/Plasma Spike-In Control as described in Appendix B, page 25.
- Use of carrier RNA (e.g., 1 µg MS2 RNA, Roche, cat. no. 10 165 948 001) or bacterial ribosomal RNA (Roche, cat. no. 10 206 938 001) may increase recovery in some cases. Do not use polyA RNA.

Procedure

1. **Prepare serum or plasma or thaw frozen samples.**
2. **Add 5 volumes QIAzol Lysis Reagent (see Table 2 for guidelines). Mix by vortexing or pipetting up and down.**

Table 2. QIAzol Lysis Reagent volumes for various serum/plasma volumes

Serum/plasma (µl)	Protocol step 2: QIAzol Lysis Reagent (µl)	Protocol step 5: chloroform (µl)	Protocol step 7: approx. volume of upper aqueous phase (µl)	Protocol step 8: 100% ethanol (µl)
≤50	250	50	150	225
100	500	100	300	450
200	1000	200	600	900

Note: If the volume of plasma or serum is not limited, we recommend using 100–200 µl per RNA preparation.

Note: After addition of QIAzol Lysis Reagent, lysates can be stored at –70°C for several months.

3. **Place the tube containing the lysate on the benchtop at room temperature (15–25°C) for 5 min.**
4. **Add 3.5 µl miRNeasy Serum/Plasma Spike-In Control (1.6 x 10^8 copies/µl working solution) and mix thoroughly.**

   For details on making appropriate stocks and working solutions of miRNeasy Serum/Plasma Spike-In Control, see Appendix B, page 25.

5. **Add chloroform of an equal volume to the starting sample to the tube containing the lysate and cap it securely (see Table 2 for guidelines). Vortex or shake vigorously for 15 s.**

   Thorough mixing is important for subsequent phase separation.

6. **Place the tube containing the lysate on the benchtop at room temperature (15–25°C) for 2–3 min.**

7. **Centrifuge for 15 min at 12,000 x *g* at 4°C. After centrifugation, heat the centrifuge up to room temperature (15–25°C) if the same centrifuge will be used for the next centrifugation steps.**

   After centrifugation, the sample separates into 3 phases: an upper, colorless, aqueous phase containing RNA; a white interphase; and a lower, red, organic phase. See Table 2 for the approximate volume of the aqueous phase.

8. **Transfer the upper aqueous phase to a new collection tube (not supplied). Avoid transfer of any interphase material. Add 1.5 volumes of 100% ethanol and mix thoroughly by pipetting up and down several times. Do not centrifuge. Continue without delay with step 9.**

   A precipitate may form after addition of ethanol, but this will not affect the procedure.

9. **Pipet up to 700 µl of the sample, including any precipitate that may have formed, into an RNeasy MinElute spin column in a 2 ml collection tube (supplied). Close the lid gently and centrifuge at ≥8000 x *g* (≥10,000 rpm) for 15 s at room temperature (15–25°C). Discard the flow-through.***

   Reuse the collection tube in step 10.

10. **Repeat step 9 using the remainder of the sample. Discard the flow-through.***

    Reuse the collection tube in step 11.

11. **Add 700 µl Buffer RWT to the RNeasy MinElute spin column. Close the lid gently and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through.***

    Reuse the collection tube in step 12.

12. **Pipet 500 µl Buffer RPE onto the RNeasy MinElute spin column. Close the lid gently and centrifuge for 15 s at ≥8000 x *g* (≥10,000 rpm) to wash the column. Discard the flow-through.**

    Reuse the collection tube in step 13.

* Flow-through contains QIAzol Lysis Reagent or Buffer RWT and is therefore not compatible with bleach. See page 6 for safety information.

13. **Pipet 500 µl of 80% ethanol onto the RNeasy MinElute spin column. Close the lid gently and centrifuge for 2 min at ≥8000 x *g* (≥10,000 rpm) to wash the spin column membrane. Discard the collection tube with the flow-through.**

    Note: 80% ethanol should be prepared with ethanol (96–100%) and RNase-free water.

    Note: After centrifugation, carefully remove the RNeasy MinElute spin column from the collection tube so that the column does not contact the flow-through. Otherwise, carryover of ethanol will occur.

14. **Place the RNeasy MinElute spin column into a new 2 ml collection tube (supplied). Open the lid of the spin column, and centrifuge at full speed for 5 min to dry the membrane. Discard the collection tube with the flow-through.**

    To avoid damage to their lids, place the spin columns into the centrifuge with at least one empty position between columns. Orient the lids so that they point in a direction opposite to the rotation of the rotor (e.g., if the rotor rotates clockwise, orient the lids counterclockwise).

    It is important to dry the spin column membrane, since residual ethanol may interfere with downstream reactions. Centrifugation with the lids open ensures that no ethanol is carried over during RNA elution.

15. **Place the RNeasy MinElute spin column in a new 1.5 ml collection tube (supplied). Add 14 µl RNase-free water directly to the center of the spin column membrane. Close the lid gently, and centrifuge for 1 min at full speed to elute the RNA.**

    As little as 10 µl RNase-free water can be used for elution if a higher RNA concentration is required, but the yield will be reduced by approximately 20%. Do not elute with less than 10 µl RNase-free water, as the spin column membrane will not be sufficiently hydrated.

    The dead volume of the RNeasy MinElute spin column is 2 µl: elution with 14 µl RNase-free water results in a 12 µl eluate.

Appendix B: Use of the miRNeasy Serum/Plasma Spike-In Control in Serum/Plasma miRNA Profiling

There is currently no clear consensus in the research community on what should be used as a normalization control for miRNA expression profiling in a serum or plasma sample. Many researchers choose to spike a synthetic miRNA into their RNA prep to monitor RNA recovery and reverse transcription efficiency. This RNA is added to samples after the addition of denaturant (e.g., QIAzol Lysis Reagent), prior to addition of chloroform and phase separation. After real-time RT-PCR, the C_T value obtained with the assay targeting the synthetic miRNA permits normalization between samples, which can control for varying RNA purification yields and amplification efficiency. In addition, RNA recovery can be assessed by comparing the C_T value to a standard curve of the synthetic miRNA generated independently of the RNA purification procedure. QIAGEN recommends the miRNeasy Serum/Plasma Spike-In Control (cat. no. 219610) for use as an internal control for miRNA expression profiling in serum or plasma. This appendix includes details of preparation of miRNeasy Serum/Plasma Spike-In Control stock and working solution, a protocol for generating an miRNeasy Serum/Plasma Spike-In Control standard curve, and a protocol for assessing the recovery of miRNeasy Serum/Plasma Spike-In Control after RNA purification.

Note: Use of the miRNeasy Serum/Plasma Spike-In Control is not recommended for cell or tissue samples.

Preparation of miRNeasy Serum/Plasma Spike-In Control

The miRNeasy Serum/Plasma Spike-In Control is a *C. elegans* miR-39 miRNA mimic and is supplied lyophilized at 10 pmol per tube. Reconstitute by adding 300 µl RNase-free water per tube, resulting in a 2×10^{10} copies/µl stock. miRNeasy Serum/Plasma Spike-In Control stock should be stored at –80°C. For large volumes, first aliquot into smaller volumes prior to long-term storage at –80°C.

When working with miRNeasy Serum/Plasma Spike-In Control, first add 4 µl of 2×10^{10} copies/µl miRNeasy Serum/Plasma Spike-In Control stock to 16 µl RNase-free water, resulting in a 4×10^{9} copies/µl dilution. If performing purification of RNA from serum and plasma, add 2 µl of the 4×10^{9} copies/µl dilution to 48 µl RNase-free water to provide a 1.6×10^{8} copies/µl working solution. If generating a standard curve, add 2 µl of the 4×10^{9} copies/µl dilution to 78 µl RNase-free water that contains carrier RNA (e.g., 10 ng/µl MS2 [Roche, cat. no. 10 165 948 001] or bacterial ribosomal RNA [Roche, cat. no. 10 206 938 001]) to provide a 1×10^{8} copies/µl working solution. These dilutions are summarized in Table 3.

Table 3. miScript Serum/Plasma Spike-In Control dilutions

Purpose	Dilution	Concentration (copies/µl)
Stock	Add 300 µl RNase-free water to lyophilized miScript Serum/Plasma Spike-In Control (10 pmol)	2×10^{10}
Dilution	Add 4 µl stock (2×10^{10} copies/µl) to 16 µl RNase-free water	4×10^{9}
Working solution for RNA purification (page 14)	Add 2 µl of 4×10^{9} copies/µl dilution to 48 µl RNase-free water	1.6×10^{8}
Working solution for generation of standard curve (page 26)	Add 2 µl of 4×10^{9} copies/µl dilution to 78 µl RNase-free water containing 10 ng/µl MS2 (Roche, cat. no. 10 165 948 001) or bacterial ribosomal RNA (Roche, cat. no. 10 206 938 001)	1×10^{8}

Protocol: Generation of miRNeasy Serum/Plasma Spike-In Control Standard Curve

This protocol is for generating a real-time PCR standard curve of miRNeasy Serum/Plasma Spike-In Control that is independent of a serum/plasma sample and RNA purification procedure. The standard curve allows estimation of the recovery of miRNeasy Serum/Plasma Spike-In Control when it is added to a serum/plasma sample that is subsequently used for RNA purification (see protocol on page 14).

Important points before starting

- To ensure reproducibility, always use freshly prepared cDNA to generate a standard curve. Perform PCRs for generation of the standard curve and PCRs on RNA from the serum/plasma samples of interest in the same run. Do not store cDNA dilutions for later use.
- This protocol uses the following components of the miScript PCR System: Ce_miR-39_1 miScript Primer Assay (provided in the miRNeasy Serum/Plasma Kit), miScript II RT Kit, miScript SYBR Green PCR Kit. For more information, consult the *miScript PCR System Handbook* or visit www.qiagen.com/miRNA.

Appendix D: Storage, Quantification, and Determination of Quality of RNA

Storage of RNA

Purified RNA may be stored at –20°C or –70°C in RNase-free water. Under these conditions, no degradation of RNA is detectable after 1 year.

Quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A_{260}) in a spectrophotometer (see "Spectrophotometric quantification of RNA" below). For small amounts of RNA, however, it may be difficult to determine amounts photometrically. Small amounts of RNA can be accurately quantified using an Agilent® 2100 Bioanalyzer, quantitative RT-PCR, or fluorometric quantification. When purifying RNA from particularly small samples (e.g., laser-microdissected samples, or from plasma or serum), quantitative, real-time RT-PCR should be used for quantification.

Spectrophotometric quantification of RNA

To ensure significance, A_{260} readings should be greater than 0.15. An absorbance of 1 unit at 260 nm corresponds to 44 µg of RNA per ml (A_{260}=1→ 44 µg/ml). This relation is valid only for measurements at a neutral pH. Therefore, if it is necessary to dilute the RNA sample, this should be done in a buffer with neutral pH.* As discussed below (see "Purity of RNA", page 36), the ratio between the absorbance values at 260 and 280 nm gives an estimate of RNA purity.

When measuring RNA samples, be certain that cuvettes are RNase-free, especially if the RNA is to be recovered after spectrophotometry. This can be accomplished by washing cuvettes with 0.1 M NaOH, 1 mM EDTA,* followed by washing with RNase-free water (see "Solutions", page 34). Use the buffer in which the RNA is diluted to zero the spectrophotometer. An example of the calculation involved in RNA quantification is shown below:

Volume of RNA sample = 100 µl

Dilution = 10 µl of RNA sample + 490 µl of 10 mM Tris·Cl,* pH 7.0 (1/50 dilution)

Measure absorbance of diluted sample in a 1 ml cuvette (RNase-free)

A_{260} = 0.2

* When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

Concentration of RNA sample	= 44 µg/ml x A_{260} x dilution factor
	= 44 µg/ml x 0.2 x 50
	= 440 µg/ml
Total amount	= concentration x volume in milliliters
	= 440 µg/ml x 0.1 ml
	= 44 µg of RNA

Purity of RNA

The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) provides an estimate of the purity of RNA with respect to contaminants that absorb in the UV spectrum, such as protein. However, the A_{260}/A_{280} ratio is influenced considerably by pH. Since water is not buffered, the pH and the resulting A_{260}/A_{280} ratio can vary greatly. Lower pH results in a lower A_{260}/A_{280} ratio and reduced sensitivity to protein contamination.* For accurate values, we recommend measuring absorbance in 10 mM Tris·Cl, pH 7.5. Pure RNA has an A_{260}/A_{280} ratio of 1.9–2.1† in 10 mM Tris·Cl, pH 7.5. Always be sure to calibrate the spectrophotometer with the same solution used for dilution.

For determination of RNA concentration, however, we recommend dilution of the sample in a buffer with neutral pH since the relationship between absorbance and concentration (A_{260} reading of 1 = 44 µg/ml RNA) is based on an extinction coefficient calculated for RNA at neutral pH (see "Quantification of RNA", page 35).

DNA contamination

No currently available purification method can guarantee that RNA is completely free of DNA, even when it is not visible on an agarose gel. While miRNeasy Kits will remove the vast majority of cellular DNA, trace amounts may still remain, depending on the amount and nature of the sample. However, serum, plasma, and other cell-free body fluids contain very little DNA.

For analysis of very low-abundance targets, any interference by residual DNA contamination can be detected by performing real-time RT-PCR control experiments in which no reverse transcriptase is added prior to the PCR step.

To prevent any interference by DNA in gene expression analysis real-time RT-PCR applications, such as with ABI PRISM and LightCycler instruments, we recommend designing primers that anneal at intron splice junctions so that genomic DNA will not be amplified. QuantiTect Primer Assays from QIAGEN

* Wilfinger, W.W., Mackey, M., and Chomczynski, P. (1997) Effect of pH and ionic strength on the spectrophotometric assessment of nucleic acid purity. BioTechniques **22**, 474.

† Values up to 2.3 are routinely obtained for pure RNA (in 10 mM Tris·Cl, pH 7.5) with some spectrophotometers.

Appendix H: Taqman Advanced miRNA Assays User Guide

appliedbiosystems

TaqMan® Advanced miRNA Assays

USER GUIDE

Single-tube assays

for use with:
TaqMan® Advanced miRNA cDNA Synthesis Kit

Catalog Number A25576
Publication Number 100027897
Revision C

For Research Use Only. Not for use in diagnostic procedures.

ThermoFisher SCIENTIFIC

Contents

1 Product information

Product description

TaqMan® Advanced miRNA Assays are pre-formulated primer and probe sets that are designed for analysis of microRNA (miRNA) expression levels using Applied Biosystems™ real-time PCR instruments. The assays can detect and quantify the mature form of the miRNA from 1–10 ng of total RNA from tissue, or 2 µL of total RNA from serum or plasma. For more information about PCR detection with TaqMan® Advanced miRNA Assays, see page "TaqMan® Advanced miRNA Assays chemistry overview" on page 22.

The TaqMan® Advanced miRNA cDNA Synthesis Kit (Cat. No. A28007; sold separately) is required for preparing the cDNA template that is used with the TaqMan® Advanced miRNA Assays. The kit enables the analysis of:

- Multiple miRNAs from a single amplified sample.
- Samples that are limited in quantity, including serum, plasma, or other biological fluids.

This document describes procedures to prepare cDNA templates from miRNA followed by PCR amplification of the cDNA template and subsequent data analysis. In the first stage of the workflow, mature miRNAs from total RNA are modified by 1) extending the 3' end of the mature transcript through poly(A) addition, then 2) lengthening the 5' end by adaptor ligation. The modified miRNAs then undergo universal reverse transcription followed by amplification to increase uniformly the amount of cDNA for all miRNAs (miR-Amp reaction). For more information about cDNA synthesis of templates for TaqMan® Advanced miRNA Assays, see page "cDNA template preparation" on page 21.

TaqMan® Advanced miRNA Assays of interest are then used for quantification of miRNA expression levels by qPCR analysis. Predesigned TaqMan® Advanced miRNA Assays are available for most human miRNAs in miRBase (the miRNA sequence repository). For a current list of assays, go to **thermofisher.com/advancedmirna**.

Note: TaqMan® Advanced miRNA Assays are for analysis of mature miRNA only. For analysis of siRNA, or other small RNAs that are fewer than 200 bases in length go to **thermofisher.com/taqmanmirna**.

The procedures in this document are for use with TaqMan® Advanced miRNA Assays supplied in the single-tube format.

Workflow

Prepare cDNA templates

Input RNA sample

▼

"Perform the poly(A) tailing reaction" on page 12

(55 minutes)

▼

"Perform the adaptor ligation reaction" on page 13

(60 minutes)

▼

"Perform the reverse transcription (RT) reaction" on page 14

(20 minutes)

▼

"Perform the miR-Amp reaction" on page 15

(30 minutes)

▼

Perform real-time PCR

"Prepare PCR reaction plate" on page 17

▼

"Set up and run the real-time PCR instrument" on page 18

(45 minutes)

▼

"Analyze the results" on page 19

2 Prepare cDNA templates

Procedural guidelines

Guidelines for preparing cDNA templates

- Follow best practices when working with RNA samples (see "Best practices for PCR and RT-PCR experiments" on page 24).
- Keep the TaqMan® Advanced miRNA Assays in storage until ready for use.
- Calculate the number of required reactions. Scale reaction components based on the single-reaction volumes, then include 10% overage.
- If using strip tubes, change to a new cap after each step or incubation.
- If using 0.2- or 0.5-mL PCR tubes, use a thermal cycler tray.
- If using plates, use a thermal cycler compression pad.

Guidelines for RNA input

- Prepare samples using a total RNA isolation method that preserves small RNAs. See Table 4 on page 8 for recommended RNA isolation kits.
- For tissue samples: Use 1–10 ng of total RNA per reaction.

  Note: Sample concentration before adding to reactions should be ≤ 5 ng/µL.
- For blood, serum, or plasma samples: Use 2 µL of sample eluent (from the sample isolation procedure) per reaction.
- For optimal reverse transcription, input RNA should be:
  - Free of inhibitors of reverse transcription (RT) and PCR
  - Dissolved in PCR-compatible buffer
  - Free of RNase activity
  - Nondenatured total RNA (not applicable for double-stranded templates)

  IMPORTANT! Do not denature the total RNA.

Perform the poly(A) tailing reaction

1. Thaw samples and cDNA synthesis reagents on ice, gently vortex to thoroughly mix, then centrifuge briefly to spin down the contents and eliminate air bubbles.

   Note: Keep the assays in storage until ready for use.

   IMPORTANT! The 50% PEG 8000 reagent must be at room temperature for the adaptor ligation reaction (next section).

2. In a 1.5-mL microcentrifuge tube, prepare sufficient Poly(A) Reaction Mix for the required number of reactions according to the following table.

Component	1 Rxn	4 Rxns[1]	10 Rxns[1]
10X Poly(A) Buffer	0.5 µL	2.2 µL	5.5 µL
ATP	0.5 µL	2.2 µL	5.5 µL
Poly(A) Enzyme	0.3 µL	1.3 µL	3.3 µL
RNase-free water	1.7 µL	7.5 µL	18.7 µL
Total Poly(A) Reaction Mix volume	**3.0 µL**	**13.2 µL**	**33 µL**

[1] Volumes include 10% overage.

3. Vortex the Poly(A) Reaction Mix to thoroughly mix the contents, then centrifuge briefly to spin down the contents and eliminate air bubbles.

4. Add 2 µL of sample to each well of a reaction plate or each reaction tube, then transfer 3 µL of Poly(A) Reaction Mix to each well or tube.

   Note: (*Optional*) Before adding the sample to the reaction plate or tube, add RNase Inhibitor Protein to each sample to minimize the effects of RNase contamination. For detailed instructions, see the documentation provided by the RNase Inhibitor Protein manufacturer.

   The total volume should be 5 µL per well or tube.

5. Seal the reaction plate or tubes, then vortex briefly to thoroughly mix the contents.

6. Centrifuge the reaction plate or tubes briefly to spin down the contents and eliminate air bubbles.

7. Place the reaction plate or tubes into a thermal cycler, then incubate using the following settings and standard cycling:

Step	Temperature	Time
Polyadenylation	37°C	45 minutes
Stop reaction	65°C	10 minutes
Hold	4°C	Hold

8. Proceed immediately to the adaptor ligation reaction (next section).

Perform the adaptor ligation reaction

1. In a 1.5-mL microcentrifuge tube, prepare sufficient Ligation Reaction Mix for the required number of reactions according to the following table.

Component	1 Rxn	4 Rxns[1]	10 Rxns[1]
5X DNA Ligase Buffer	3 µL	13.2 µL	33 µL
50% PEG 8000[2]	4.5 µL	19.8 µL	49.5 µL
25X Ligation Adaptor	0.6 µL	2.6 µL	6.6 µL
RNA Ligase	1.5 µL	6.6 µL	16.5 µL
RNase-free water	0.4 µL	1.8 µL	4.4 µL
Total Ligation Reaction Mix volume	**10 µL**	**44 µL**	**110 µL**

[1] Volumes include 10% overage.
[2] 50% PEG 8000 is very viscous, follow the Important statement below to ensure accurate pipetting.

IMPORTANT! For accurate pipetting of 50% PEG 8000:

- Use 50% PEG 8000 at room temperature.
- Aspirate and dispense solution slowly.
  a. Hold the pipette tip in the solution for ~10 seconds after releasing the plunger during aspiration. This action allows the solution to be fully drawn into the pipette tip.
  b. Keep the plunger depressed for ~10 seconds to allow the solution to be fully dispensed into the Ligation Reaction Mix.

2. Vortex the Ligation Reaction Mix to thoroughly mix the contents, then centrifuge briefly to spin down the contents and eliminate air bubbles.

3. Transfer 10 µL of the Ligation Reaction Mix to each well of the reaction plate or each reaction tube containing the poly(A) tailing reaction product.
   The total volume should be 15 µL per well or tube.

4. Seal the reaction plate or tubes, then vortex briefly or shake (1,900 rpm for 1 minute with an Eppendorf™ MixMate™) to thoroughly mix the contents.

   IMPORTANT! Watch for a swirling motion of the adaptor ligation reaction to ensure proper mixing, which is necessary for efficient ligation.

5. Centrifuge the reaction plate or tubes briefly to spin down the contents.

6. Place the reaction plate or tubes into a thermal cycler, then incubate using the following settings and standard cycling:

Step	Temperature	Time
Ligation	16°C	60 minutes
Hold	4°C	Hold

7. Proceed immediately to the reverse transcription (RT) reaction (next section).

Perform the reverse transcription (RT) reaction

1. In a 1.5-mL microcentrifuge tube, prepare sufficient RT Reaction Mix for the required number of reactions according to the following table.

Component	1 Rxn	4 Rxns[1]	10 Rxns[1]
5X RT Buffer	6 µL	26.4 µL	66 µL
dNTP Mix (25 mM each)	1.2 µL	5.3 µL	13.2 µL
20X Universal RT Primer	1.5 µL	6.6 µL	16.5 µL
10X RT Enzyme Mix	3 µL	13.2 µL	33 µL
RNase-free water	3.3 µL	14.5 µL	36.3 µL
Total RT Reaction Mix volume	**15 µL**	**66 µL**	**165 µL**

[1] Volumes include 10% overage.

2. Vortex the RT Reaction Mix to thoroughly mix the contents, then centrifuge briefly to spin down the contents and eliminate air bubbles.
3. Transfer 15 µL of the RT Reaction Mix to each well of the reaction plate or each reaction tube containing the adaptor ligation reaction product.
   The total volume should be 30 µL per well or tube.
4. Seal the reaction plate or tubes, then vortex briefly to thoroughly mix the contents.
5. Centrifuge the reaction plate or tubes briefly to spin down the contents.
6. Place the reaction plate or tubes into a thermal cycler, then incubate using the following settings and standard cycling:

Step	Temperature	Time
Reverse transcription	42°C	15 minutes
Stop reaction	85°C	5 minutes
Hold	4°C	Hold

7. Proceed to the miR-Amp reaction (next section).

Store the RT reaction product at –20°C for up to 2 months.

Perform the miR-Amp reaction

1. In a 1.5-mL microcentrifuge tube, prepare sufficient miR-Amp Reaction Mix for the required number of reactions according to the following table.

Component	1 Rxn	4 Rxns[1]	10 Rxns[1]
2X miR-Amp Master Mix	25 µL	110 µL	275 µL
20X miR-Amp Primer Mix	2.5 µL	11 µL	27.5 µL
RNase-free water	17.5 µL	77 µL	192.5 µL
Total miR-Amp Reaction Mix volume	**45 µL**	**198 µL**	**495 µL**

[1] Volumes include 10% overage.

2. Vortex the miR-Amp Reaction Mix to thoroughly mix the contents, then centrifuge briefly to spin down the contents and eliminate air bubbles.
3. Transfer 45 µL of the miR-Amp Reaction Mix to each well of a *new* reaction plate or reaction tube.
4. Add 5 µL of the RT reaction product to each reaction well or each reaction tube. The total volume should be 50 µL per well or tube.
5. Seal the reaction plate or tubes, then vortex briefly to thoroughly mix the contents.
6. Centrifuge the reaction plate or tubes briefly to spin down the contents.
7. Place the reaction plate or tubes into a thermal cycler, then incubate using the following settings, MAX ramp speed, and standard cycling:

Step	Temperature	Time	Cycles
Enzyme activation	95°C	5 minutes	1
Denature	95°C	3 seconds	14
Anneal/Extend	60°C	30 seconds	
Stop reaction	99°C	10 minutes	1
Hold	4°C	Hold	1

8. Proceed to performing the real-time PCR (next section).

Store the undiluted miR-Amp reaction product at –20°C for up to 2 months.

3 Perform real-time PCR

Procedural guidelines for performing real-time PCR

- Follow best practices when performing PCR reactions (see "Best practices for PCR and RT-PCR experiments" on page 24).
- Prepare the real-time PCR reactions in an area free of artificial templates and siRNA transfections. High-copy-number templates can easily contaminate the real-time PCR reactions.
- Keep the assays protected from light and stored at –20°C until ready for use. Excessive exposure to light may affect the fluorescent probe.
- Configure plate documents according to the instructions provided in the real-time PCR instrument resource documents.
- Prepare the real-time PCR Reaction Mix before transferring it to the reaction plate for thermal cycling and fluorescence analysis.
- We recommend four replicates of each reaction.
- Calculate the number of required reactions. Scale reaction components based on the single-reaction volumes, then include 10% overage.
- Reaction volumes listed in this section are for 20-µL PCR reactions.
- For reaction volumes that are different from those detailed, scale all components proportionally.
  Reaction volumes < 10 µL are not recommended.

Prepare PCR reaction plate

1. Thaw the assays on ice, gently vortex to thoroughly mix, then centrifuge briefly to spin down the contents and eliminate air bubbles.
2. Prepare 1:10 dilution of cDNA template.
   For example, add 5 µL of the miR-Amp reaction product to 45 µL 0.1X TE buffer.
3. Gently shake the bottle of TaqMan® Fast Advanced Master Mix to thoroughly mix the contents. Do not invert the bottle.
4. In a 1.5-mL microcentrifuge tube, prepare sufficient PCR Reaction Mix for the required number of reactions according to the following table.

Component	1 Rxn	4 Rxns[1]
TaqMan® Fast Advanced Master Mix (2X)	10 µL	44.0 µL
TaqMan® Advanced miRNA Assay (20X)	1 µL	4.4 µL
RNase-free water	4 µL	17.6 µL
Total PCR Reaction Mix volume	**15 µL**	**66 µL**

[1] Volumes include 10% overage.

5. Vortex the PCR Reaction Mix to thoroughly mix the contents, then centrifuge briefly to spin down the contents and eliminate air bubbles.
6. Transfer 15 µL of the PCR Reaction Mix to each well of a PCR reaction plate.
7. Add 5 µL of the diluted cDNA template to each reaction well of the plate.
   The total volume should be 20 µL per reaction well.
8. Seal the reaction plate with an adhesive cover, then vortex briefly to thoroughly mix the contents.
9. Centrifuge the reaction plate briefly to spin down the contents.

Set up and run the real-time PCR instrument

See the appropriate instrument user guide for detailed instructions to program the thermal-cycling conditions or to run the plate.

The following thermal profiles are optimized for use with TaqMan® Fast Advanced Master Mix and can be used with Fast or Standard reaction plates and the corresponding instrument block configurations.

1. Load the reaction plate in the real-time PCR instrument.
2. Set the appropriate experiment settings and PCR thermal cycling conditions for your instrument. Select the fast cycling mode for all instruments.

Table 7 StepOnePlus™, ViiA™ 7, and QuantStudio™ systems

Step	Temperature	Time	Cycles
Enzyme activation	95°C	20 seconds	1
Denature	95°C	1 second	40
Anneal / Extend	60°C	20 seconds	

Table 8 7500 and 7500 Fast systems

Step	Temperature	Time	Cycles
Enzyme activation	95°C	20 seconds	1
Denature	95°C	3 seconds	40
Anneal / Extend	60°C	30 seconds	

3. Set the reaction volume appropriate for the reaction plate.
4. Start the run.

Analyze the results

For detailed information about data analysis, see the appropriate documentation for your instrument. Use the standard curve method or the relative quantification ($\Delta\Delta C_t$) method to analyze results.

The general guidelines for analysis include:

- View the amplification plot; then, if needed:
  - Adjust the baseline and threshold values.

    Note: A threshold value of 0.1 is recommended.
  - Remove outliers from the analysis.
- In the well table or results table, view the C_t values for each well and for each replicate group.

Analyze data generated with TaqMan® Advanced miRNA Assays using any of the following tools:

Software	Resource
Applied Biosystems™ real-time PCR Analysis Modules	thermofisher.com/us/en/home/cloud.html
ExpressionSuite™ Software	thermofisher.com/us/en/home/technical-resources/software-downloads/expressionsuite-software.html

For more information about real-time PCR, go to: **thermofisher.com/qpcreducation**.

Appendix I: Non-expressed and Expressed Samples in miR-100 and -133a

Table 22. Comparison between Non-expressed and Expressed Samples of miR-100

Variable		Non-Express (n=39)	Express (n=24)	*P*
Age (yr)		67.9 ± 5.7	72.2 ± 5.6	0.005
Height (cm)		164.4 ± 5.5	160.2 ± 7.6	0.026
Total aBMC (g)		2410.9 ± 368.4	2169.5 ± 296.0	0.009
JHt (inch)		7.8 ± 2.2	6.6 ± 1.9	0.030
Ca^{2+} (mg/day)		1367.2 ± 614.8	1780.6 ± 830.7	0.027
BPAQ Past		104.2 ± 93.0	56.3 ± 41.8	0.021
BPAQ Total		54.1 ± 46.7	29.6 ± 21.7	0.019
Tot vBMC (mg/mm)	38%	304.5 ± 33.4	282.7 ± 25.7	0.008
Cort vBMC (mg/mm)		289.1 ± 34.2	266.6 ± 27.8	0.009
Cort Area (mm^2)		248.3 ± 30.7	230.2 ± 22.5	0.015
SSI (mm^3)		1405.4 ± 210.5	1298.7 ± 194.2	0.049
Total vBMC (mg/mm)	66%	329.4 ± 40.6	299.1 ± 33.5	0.003
Cort vBMC (mg/mm)		289.8 ± 40.0	260.2 ± 41.7	0.008
Cort Area (mm^2)		260.4 ± 36.0	235.4 ± 31.8	0.007
Ipolar (mm^4)		36167 ± 7215	32008 ± 6003	0.021
SSI (mm^3)		2076.5 ± 317.4	1858.5 ± 260.4	0.006

Mean ± SD; JHt: Jump Height; Ca^{2+}: Calcium Intake; Tot: Total; Cort: Cortical; SSI: Stress Strain Index

Table 23. Comparison between Non-expressed and Expressed Samples of miR-133a

Variable		Non-Express (n=49)	Express (n=14)	*P*
Age (yr)		68.8 ± 6.2	72.3 ± 4.5	0.005
Total aBMD (g/cm^2)		1.139 ± 0.103	1.078 ± 0.081	0.009
JHt (inch)		7.7 ± 2.1	6.0 ± 2.1	0.030
BPAQ Past		97.7 ± 85.2	44.8 ± 43.1	0.021
BPAQ Total		50.7 ± 42.8	23.9 ± 22.9	0.019
Tot vBMD (mg/cm^3)	4%	275.3 ± 34.4	242.7 ± 42.6	0.004
Tot Area (mm^2)		965.7 ± 101.1	1056.1 ± 138.5	0.004
Trab vBMD (mg/cm^3)		237.5 ± 31.6	207.4 ± 40.3	0.036
Trab Area (mm^2)		796.7 ± 90.7	877.5 ± 123.0	0.036
Peri_C (mm)		110.0 ± 5.8	115.0 ± 7.7	0.039
Tot BSI (mg^2/mm^4)		73.9 ± 17.3	62.5 ± 18.7	0.037
Trab BSI (mg^2/mm^4)		45.6 ± 12.0	38.1 ± 13.0	0.047
Tot vBMD (g/cm^3)	38%	882.1 ± 73.2	818.4 ± 77.0	0.006
Cort vBMC (mg/mm)		285.0 ± 33.1	264.7 ± 31.4	0.046
Endo_C (mm)		34.5 ± 4.6	38.0 ± 5.0	0.018
Tot vBMD (g/cm^3)	66%	642.7 ± 81.4	580.2 ± 103.4	0.020
Cort vBMC (mg/mm)		284.3 ± 41.1	258.5 ± 44.2	0.045

Mean ± SD; JHt: Jump Height; Tot: Total; Trab: Trabecular; Peri_C: Periosteal Circumference; Cort: Cortical; Endo_C: Endosteal Circumference