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# Melatonin-micronutrients Osteopenia Treatment Study (MOTS): A translational study assessing the effects of melatonin, strontium citrate, vitamin D3 and vitamin K2 on bone density, bone turnover markers and health-related quality of life in postmenopausal osteopenic women following a one-year double-blind randomized placebo-controlled trial and on osteoblast-osteoclast co-cultures

Sifat Maria

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MELATONIN-MICRONUTRIENTS OSTEOGENESIS TREATMENT STUDY (MOTS): A  
TRANSLATIONAL STUDY ASSESSING THE EFFECTS OF MELATONIN, STRONTIUM  
CITRATE, VITAMIN D<sub>3</sub> AND VITAMIN K<sub>2</sub> ON BONE DENSITY, BONE TURNOVER  
MARKERS AND HEALTH-RELATED QUALITY OF LIFE IN POSTMENOPAUSAL  
OSTEOPENIC WOMEN FOLLOWING A ONE-YEAR DOUBLE-BLIND RANDOMIZED  
PLACEBO-CONTROLLED TRIAL AND ON OSTEOBLAST-OSTEOCLAST CO-  
CULTURES

A Dissertation

Submitted to the Graduate School of Pharmaceutical Sciences

Duquesne University

In partial fulfillment of the requirements for  
the degree of Doctor of Philosophy

By

Sifat Maria

May 2018

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Sifat Maria

2018

MELATONIN-MICRONUTRIENTS OSTEOPENIA TREATMENT STUDY (MOTS): A TRANSLATIONAL STUDY ASSESSING THE EFFECTS OF MELATONIN, STRONTIUM CITRATE, VITAMIN D<sub>3</sub> AND VITAMIN K<sub>2</sub> ON BONE DENSITY, BONE MARKERS TURNOVER AND HEALTH-RELATED QUALITY OF LIFE IN POSTMENOPAUSAL OSTEOPENIA FOLLOWING A ONE-YEAR DOUBLE-BLIND RANDOMIZED PLACEBO-CONTROLLED TRIAL AND ON OSTEOLAST-OSTEOCLAST CO-CULTURES.

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## ABSTRACT

MELATONIN-MICRONUTRIENTS OSTEOPENIA TREATMENT STUDY (MOTS): A TRANSLATIONAL STUDY ASSESSING THE EFFECTS OF MELATONIN, STRONTIUM CITRATE, VITAMIN D<sub>3</sub> AND VITAMIN K<sub>2</sub> ON BONE DENSITY, BONE MARKERS TURNOVER AND HEALTH-RELATED QUALITY OF LIFE IN POSTMENOPAUSAL OSTEOPENIA FOLLOWING A ONE-YEAR DOUBLE-BLIND RANDOMIZED PLACEBO-CONTROLLED TRIAL AND ON OSTEOBLAST-OSTEOCLAST CO-CULTURES.

By

Sifat Maria

May 2018

Dissertation supervised by Dr. Paula A. Witt-Enderby

**Objective:** The purpose of this study was to assess if a novel combination of melatonin and three other natural bone-aiding micronutrients: strontium citrate, vitamins D<sub>3</sub> and K<sub>2</sub> (MSDK) could improve bone health by modulating the activity of osteoblasts and osteoclasts in favor of balanced bone remodeling and by improving the overall health-related quality of life in postmenopausal osteopenic women.

**Methods:** The Melatonin-micronutrients Osteopenia Treatment Study (MOTS) is a translational research study that used both clinical and *in vitro* approaches to assess the efficacy of MSDK on bone health in women and to identify potential mechanisms for its effects. The clinical component of this study was designed as a one-year double-blind, placebo-controlled randomized trial, which assessed the effects of nightly MSDK supplementation containing 5 mg melatonin, 450 mg strontium citrate, 2000 IU vitamin D<sub>3</sub> and 60 mcg vitamin K<sub>2</sub> (MK7) on bone mineral density (BMD), bone marker turnover and quality of life (QOL) in postmenopausal osteopenic women. A total of 22 women (ages 49–75) were randomized to receive either MSDK (n = 11) or placebo (n

= 11) p.o. nightly for 12 months. Bone mineral density (BMD) was measured by dual-energy X-ray absorptiometry (DXA) and Achilles ultrasound. Bone turnover markers total procollagen type I amino-terminal propeptide (P1NP), osteocalcin (OC; both intact and N-terminal mid-fragments) and collagen type I c-telopeptide (CTX) were assessed at months 0, 6 and 12 in serum. Participants' serum vitamin D<sub>3</sub> and C-reactive protein (CRP) levels were measured at months 0, 6 and 12. Nocturnal urinary melatonin levels were measured at month 12. Quality of life questionnaires measuring menopausal symptoms (MENQOL), anxiety (STAI), stress (PSS) and depression (CES-D) were administered at months 0, 6 and 12. Participants were given a daily diary to keep track of their pill intake, sleep duration, exercise, supplement usage and other information relevant to their general health and mood throughout the study.

The *in vitro* component of this translational study focused on identifying potential mechanisms underlying MSDK's effect on bone cell differentiation and activity using two co-culture systems containing human adult mesenchymal stem cells (hMSCs) and human peripheral blood monocytes (hPBMCs). Using a novel *in vitro* treatment paradigm that closely mimics the *in vivo* condition, hMSCs/hPBMCs were co-cultured for 21 days either separately using transwell culture dishes (transwell co-culture) or by seeding hPBMCs directly on top of differentiating hMSCs (layered co-culture). The effect of MSDK on the differentiation and activity of bone cells was measured via alizarin red staining assay for osteoblast activity and TRAP and resorption pit assays for osteoclast activity, respectively. This study further assessed various signaling cascades underlying MSDK's effects on osteoblastogenesis and osteoclastogenesis that included: OPG/RANKL, ERK1/2 and 5, RUNX2, INTEGRIN  $\beta$ 1, NF $\kappa$ B, PPAR $\gamma$ , GLUT4 and INSULIN R $\beta$ .

**Results:** One-year of MSDK treatment significantly increased lumbar spine BMD (4.3%), left femoral neck BMD (2.2%), with an upward trend for total left hip BMD (5.03% vs. 2.2% in placebo;  $p=.069$ ) in postmenopausal osteopenic women taking MSDK compared to placebo. MSDK also decreased the ten-year probability of vertebral fracture risk by 6.48% compared to the 10.8% increase observed in placebo. MSDK reduced bone turnover ( $\downarrow$ CTx:P1NP ratio) primarily by increasing the serum bone formation marker P1NP (vs. placebo;  $p = 0.023$  and  $p = 0.004$  at months 6 and 12, respectively); the bone resorption marker, CTx remained constant throughout the study. Serum OC levels also did not change with MSDK throughout the study. Serum CRP levels showed a downward trend, suggesting potentially positive effects of MSDK on one's inflammatory status. MSDK produced no effect on height, weight and lean body mass; however, MSDK resulted in less variability in weight gain or loss compared to women taking placebo which could positively contribute to bone health. MSDK exhibited beneficial effects on the quality of life, perhaps by lessening the sexual symptoms of menopause (not significant vs. placebo) and showing some improvements with respect to sleep quality. MSDK did not produce adverse effects psychologically or physically in our cohort and there was a high compliance rate (92.4%).

MSDK-exposed human mesenchymal stem cells (hMSCs) and human peripheral blood monocytes (hPBMCs) plated in transwells or layered co-cultures demonstrated increases in osteoblastogenesis, decreases in osteoclastogenesis, increases in the ratio of OPG:RANKL by both increasing OPG and decreasing RANKL expression in osteoblasts. In transwell osteoblasts, MSDK increased pERK1/2 and RUNX2 levels; decreased ERK5; and did not affect the expression of NF $\kappa$ B and INTEGRIN  $\beta$ 1. In layered osteoblasts, MSDK also decreased expression of the metabolic proteins PPAR $\gamma$  and GLUT4. These findings demonstrate that MSDK may be a novel, safe and efficacious therapy for treating those afflicted with osteopenia.

## DEDICATION

To my parents Md. Shafiqur Rahman and Noorjahan Begum, who sowed the beautiful dream of pursuing Ph.D. in me and made me who I am today.

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Graduate life was an amazing journey for me. There were ups and downs, joys and pains, but when I look back, I feel like it's one of the most worthwhile endeavors I have ever taken. It's not only because of the degree and training I have earned, but also because of the people I have met, because of their care, guidance, support and constant encouragement. I would like to take this opportunity to express my sincere gratitude to all these wonderful people.

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Next, I would like to express my sincere gratitude to my committee members, Dr. David Johnson, Dr. Lauren O'Donnell, Dr. Frank D'Amico and Dr. Holly Lassila. Their valuable suggestions as well as constructive criticism greatly enhanced the productivity of this dissertation work. I would like to give special thanks to Dr. D'Amico for giving me statistical lessons with great care and patience despite his busy schedule and helping me design the clinical study. I am also thankful to other pharmacology faculties at Duquesne University, specially Dr. Rehana Leak and Dr. Jane Cavanaugh, for their academic guidance and valuable contribution in improving my knowledge and technical skills. I would like to thank our collaborators- Dr. Mark Swanson for

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## TABLE OF CONTENTS

|  |             |
|--|-------------|
| <b>ABSTRACT</b> .....  | <b>iv</b>   |
| <b>DEDICATION</b> .....  | <b>vii</b>  |
| <b>ACKNOWLEDGEMENTS</b> .....  | <b>viii</b> |
| <b>LIST OF FIGURES</b> .....   | <b>xiv</b>  |
| <b>LIST OF TABLES</b> .....  | <b>xv</b>   |
| <b>Chapter 1: Introduction</b> .....   | <b>1</b>    |
| 1.1. Bone physiology .....   | 1           |
| 1.1.1. Modeling and remodeling .....   | 1           |
| 1.1.2. Role of osteoblast-osteoclast communication in bone remodeling.....   | 2           |
| 1.2 Bone loss- Osteopenia and osteoporosis.....  | 6           |
| 1.2.1. Introduction to osteopenia and osteoporosis.....  | 6           |
| 1.2.2. Risk factors for osteopenia and osteoporosis.....   | 7           |
| 1.2.3. Prevalence of osteopenia and osteoporosis.....  | 9           |
| 1.3. Menopause and bone loss .....   | 11          |
| 1.4. Bone loss therapy—current and future perspectives .....   | 13          |
| 1.5. Research objective .....  | 16          |
| 1.6. Effects of melatonin, strontium citrate, vitamin D <sub>3</sub> and vitamin K <sub>2</sub> on bone .....  | 17          |
| 1.6.1. Melatonin .....   | 17          |
| 1.6.2. Strontium.....  | 20          |
| 1.6.3. Vitamin D <sub>3</sub> .....  | 23          |
| 1.6.4. Vitamin K <sub>2</sub> .....  | 23          |
| 1.7. Rationale for choosing melatonin, strontium citrate, vitamin D <sub>3</sub> and K <sub>2</sub> (MSDK) .....   | 24          |
| 1.8. Hypothesis.....   | 29          |
| 1.9. Specific aims.....  | 29          |
| <b>Chapter 2: MOTS clinical trial— Assessing the effects of melatonin, strontium citrate, vitamin D<sub>3</sub> (cholecalciferol) and vitamin K<sub>2</sub> (MK7) on bone health (bone density, bone markers turnover and fracture risk) and health-related quality of life in postmenopausal osteopenia</b> ..... | <b>30</b>   |
| 2.1. Materials and Methods.....  | 30          |
| 2.1.1. Recruitment and enrollment.....   | 30          |
| 2.1.2. Randomization and treatment allocation .....  | 36          |
| 2.1.3. Treatment regimen .....   | 37          |
| 2.1.4. Treatment follow-up .....   | 39          |



|   |            |
|---|------------|
| 2.1.5. Participants' right and confidentiality of the study.....  | 39         |
| 2.1.6. Bone density measurement .....   | 41         |
| 2.1.7. Fracture risk assessment .....   | 42         |
| 2.1.8. Collection and storage of serum and urine samples.....   | 44         |
| 2.1.9. Biochemical assessments .....  | 45         |
| 2.1.10. Blood pressure and body composition.....  | 47         |
| 2.1.11. Psychometric analyses .....   | 48         |
| 2.1.12. General well-being and treatment compliance as measured by diary logs.....  | 54         |
| 2.1.13. Statistical interpretations.....  | 57         |
| 2.2. Results.....   | 58         |
| 2.2.1. Participant's recruitment and enrollment.....  | 58         |
| 2.2.2. Similar baseline characteristics between groups ensured efficient randomization.....   | 61         |
| 2.2.3. MSDK improved bone health in postmenopausal women .....  | 66         |
| 2.2.4. One-year MSDK supplementation increased nocturnal melatonin levels, but did not change serum vitamin D <sub>3</sub> levels in postmenopausal women .....   | 71         |
| 2.2.5. MSDK had possible declination effect on serum C-reactive protein (CRP) .....   | 73         |
| 2.2.6. MSDK did not affect morphometric parameters and blood pressure, favoring bone health in postmenopausal women .....   | 74         |
| 2.2.7. MSDK had no worsening effect on psychometric parameters in postmenopausal women.....   | 78         |
| 2.2.8. MSDK improved general well-being and compliance in postmenopausal women....  | 83         |
| 2.3. Discussion.....  | 86         |
| <b>Chapter 3: Assessment of mechanisms underlying the effect of melatonin, strontium citrate, vitamin D<sub>3</sub> and vitamin K<sub>2</sub> (MSDK) on human adult mesenchymal stem cells and human peripheral blood monocytes grown as co-cultures.....</b> | <b>102</b> |
| 3.1. Materials and Methods.....   | 102        |
| 3.1.1. <i>In-vitro</i> treatment preparation .....  | 102        |
| 3.1.2. Osteoblast/Osteoclast co-cultures and hMSC mono-cultures.....  | 102        |
| 3.1.3. Osteoblast differentiation and mineralization.....   | 107        |
| 3.1.4. Osteoclast differentiation and resorption pit formation .....  | 107        |
| 3.1.5. Western blot.....  | 109        |
| 3.1.6. Measurement of secreted OPG and RANKL.....   | 110        |
| 3.1.7. Statistical interpretation .....   | 111        |
| 3.2. Results.....   | 111        |
| 3.2.1. MSDK increased osteoblastogenesis and decreased osteoclastogenesis in co-cultures of hMSCs and hPBMCs.....   | 111        |

|  |            |
|--|------------|
| 3.2.2. MSDK modulates OPG and RANKL levels in co-cultures of hMSCs and hPBMCs dependent upon the type of culturing condition—layered or transwell.....           | 119        |
| 3.2.3. MSDK modulates pERK1/2 and pERK5 levels in co-cultures of hMSCs and hPBMCs dependent upon the type of culturing condition—layered or transwell.....       | 123        |
| 3.2.4. MSDK modulates RUNX2 level in co-cultures of hMSCs and hPBMCs dependent upon the type of culturing condition—layered or transwell .....                   | 127        |
| 3.2.5. MSDK did not modulate INTEGRIN $\beta$ 1 level in co-cultures of hMSCs and hPBMCs .....   | 128        |
| 3.2.6. MSDK modulates NF $\kappa$ B level in co-cultures of hMSCs and hPBMCs dependent upon the type of culturing condition—layered or transwell .....           | 129        |
| 3.2.7. MSDK modulates PPAR $\gamma$ and GLUT4 levels in co-cultures of hMSCs and hPBMCs dependent upon the type of culturing condition—layered or transwell..... | 131        |
| 3.3. Discussion.....   | 132        |
| <b>Chapter 4: Strength and limitations .....</b>   | <b>150</b> |
| <b>Chapter 5: Conclusions .....</b>  | <b>152</b> |
| <b>Chapter 6: References .....</b>   | <b>157</b> |
| <b>Chapter 7: Appendix .....</b>   | <b>173</b> |
| I. The study information in <i>ClinicalTrials.gov</i> .....  | 173        |
| II. Materials used for study advertisements .....  | 176        |
| III. Initial visit form .....  | 182        |
| IV. Study medication label .....   | 183        |
| V. Treatment effects on bone density T-scores changes in one year.....   | 184        |
| VI. Treatment effects on bone marker changes in every six months.....  | 185        |
| VII. Bone markers (P1NP, OC and CTx) of participants enrolled in fall and spring.....  | 187        |
| <b>COPYRIGHT STATEMENT.....</b>  | <b>188</b> |

## LIST OF FIGURES

|   |     |
|---|-----|
| Figure 1. Different phases of bone remodeling cycle .....   | 4   |
| Figure 2: Different stages of bone loss .....   | 7   |
| Figure 3. Regulation of signaling pathways in osteoblast and osteoclast and bone remodeling by melatonin (Mel), strontium (Sr), vitamins D <sub>3</sub> and K <sub>2</sub> . 2014 ..... | 27  |
| Figure 4. Baseline intake form .....  | 34  |
| Figure 5. Study medications (MSDK and Placebo) in identical bottles .....   | 38  |
| Figure 6. Fracture risk (FRAX®) assessment questionnaire .....  | 43  |
| Figure 7. Fracture risk (FRAX®) assessment tool .....   | 44  |
| Figure 8. Menopause Specific Quality of Life (MENQOL) questionnaire .....   | 51  |
| Figure 9. Perceived Stress Scale (PSS) questionnaire .....  | 53  |
| Figure 10. Daily diary .....  | 56  |
| Figure 11. Responses to recruitment strategies employed in the study (n=184) .....  | 58  |
| Figure 12. Flow diagram of study subjects' recruitment and enrollment. ....   | 60  |
| Figure 13. Baseline characteristics of the study cohort stratified by treatment .....   | 63  |
| Figure 14. Treatment effects on bone mineral density (BMD).....   | 67  |
| Figure 15. Treatment effects on fracture risk probability (FRAX).....   | 68  |
| Figure 16. Treatment effects on serum bone markers turnover .....   | 70  |
| Figure 17. Treatment effects on urinary nocturnal melatonin and serum vitamin D <sub>3</sub> .....  | 72  |
| Figure 18. Treatment effects on serum C-reactive protein (CRP).....   | 74  |
| Figure 19. Treatment effects on body composition .....  | 76  |
| Figure 20. Treatment effects on blood pressure.....   | 77  |
| Figure 21. Treatment effects on the menopause quality of life, anxiety, stress and depression... 81   | 81  |
| Figure 22. Treatment effects on participants' (A) sleep quality, mood, GI upset and general aches/pains; (B) sleep duration and (C) exercise intensity .....                            | 85  |
| Figure 23. Isolation of monocytes (hPBMCs) from blood sample.....   | 105 |
| Figure 24. Development of hMSCs/hPBMCs transwell (indirect) and layered (direct) co-cultures. ....  | 106 |
| Figure 25. Effect of MSDK on osteoblast-mediated calcium mineralization.....  | 114 |
| Figure 26. Effect of MSDK on osteoclast differentiation and resorption pit formation.....   | 117 |
| Figure 27. Effect of MSDK on OPG and RANKL expression.....  | 122 |
| Figure 28. Effect of MSDK on MAPK (ERK1/2 and ERK5) .....   | 126 |
| Figure 29. Effect of MSDK on RUNX2. ....  | 128 |
| Figure 30. Effect of MSDK on INTEGRIN $\beta$ 1 .....   | 129 |
| Figure 31. Effect of MSDK on NF $\kappa$ B.....   | 130 |
| Figure 32. Effect of MSDK on metabolic proteins.....  | 132 |
| Figure 33. Potential mechanism underlying MSDK effects on bone formation.....   | 155 |

## LIST OF TABLES

|  |           |
|--|-----------|
| Table 1: Experimental timeline of MOTS clinical trial .....  | <b>40</b> |
| Table 2: Baseline characteristics of the study cohort un-stratified and stratified by treatment (MSDK and placebo) .....                                 | <b>62</b> |
| Table 3: Treatment effects on bone density (T-scores), fracture risk probability (FRAX), bone marker, body composition and psychometric parameters ..... | <b>82</b> |
| Table 4: Sleep duration, exercise intensity and pill intake in placebo and MSDK groups .....   | <b>86</b> |

## **Chapter 1: Introduction**

### **1.1. Bone physiology**

#### **1.1.1. Modeling and remodeling**

Vertebrates embrace a unique skeletal morphology made up of bone and cartilage. Nearly 80% of the adult human bones are comprised of compact, smooth outer layer called “cortex”; the remaining 20% are the spongy, honeycomb-like inner layer known as “trabecular” or “cancellous” bone. Cortical and trabecular bones are organized in a lamellar pattern to maintain structural rigidity ([Clarke 2008](#)). The tissue structure of bone consists of mineralized and non-mineralized matrix (osteoid) and three major bone cells—bone-forming osteoblasts, bone-lining osteocytes and bone-resorbing osteoclasts. The evolution and activity of these cells as well as communication between them are the chief controllers of skeletal development, bone adaptation (modeling) and bone preservation (remodeling) ([Gasser and Kneissel 2017](#)).

Bone modeling is the process where osteoblastic bone formation and osteoclastic bone resorption occur as two independent events. Bone modeling is predominant during the first twenty years of life at the endocortical and trabecular surface to attain peak bone mass and then continues at a low level throughout the life. Bone modeling alters bone shape or optimizes bone mass to encounter and adapt mechanical forces without bending or cracking while at the same time helps bone to resist deformation from impact loading. Thus, it primarily regulates skeletal growth and provides mechanical support during hematopoiesis and endocrine function ([Burr and Allen 2013](#), [Currey 2013](#), [Iolascon, Frizzi et al. 2014](#), [Gasser and Kneissel 2017](#)).

Once skeletal growth is accomplished, each of the adult 206 bones undergoes bone remodeling for the rest of one’s life via a tightly coupled bone formation and bone resorption process. This essential reparation process substitutes primary juvenile bones as well as age- and

stress-related microfractured bones with the new healthy, strong and more mechanically-competent bones. Bone microdamage accumulates when the magnitude of an applied load exceeds the structural strength of bone eventually leading to bone deterioration and fracture. The unique ability of bone to immaculately carry mechanical load and resist fracture is determined by bone mass and their spatial distribution (microarchitecture), which relies on the steady bone remodeling ([Clarke 2008](#), [Seeman 2008](#), [Iolascon, Frizzi et al. 2014](#), [Gasser and Kneissel 2017](#)).

### **1.1.2. Role of osteoblast-osteoclast communication in bone remodeling**

Balanced bone remodeling is a hallmark of skeletal integrity, which is defined by the balanced function of two major bone cells— osteoblasts and osteoclasts. Osteoblastic lineage cells, including mesenchymal stem cell progenitors, pre-osteoblasts, osteoblasts and osteocytes control osteoclast function which includes the fusion of monocytic pronuclei and their attachment, activity and apoptosis during bone remodeling. Osteoclasts, on the other hand, resorb bone and regulate osteoblast function by both membrane-bound and secreted factors and through the release of factors within the matrix ([Sims and Gooi 2008](#)). In cellular fos (c-fos) and macrophage colony stimulating factor (M-CSF) knockout mice, the absence of osteoclasts leads to defective bone formation, suggesting essential roles of these factors in maintaining bone integrity ([Henriksen, Neutzsky-Wulff et al. 2009](#), [Eriksen 2010](#)). Therefore, a constant flow of communication between osteoclasts and osteoblasts is essential to maintain synchronization between bone formation and resorption during bone remodeling ([Matsuo and Irie 2008](#), [Sims and Gooi 2008](#), [Raggatt and Partridge 2010](#)).

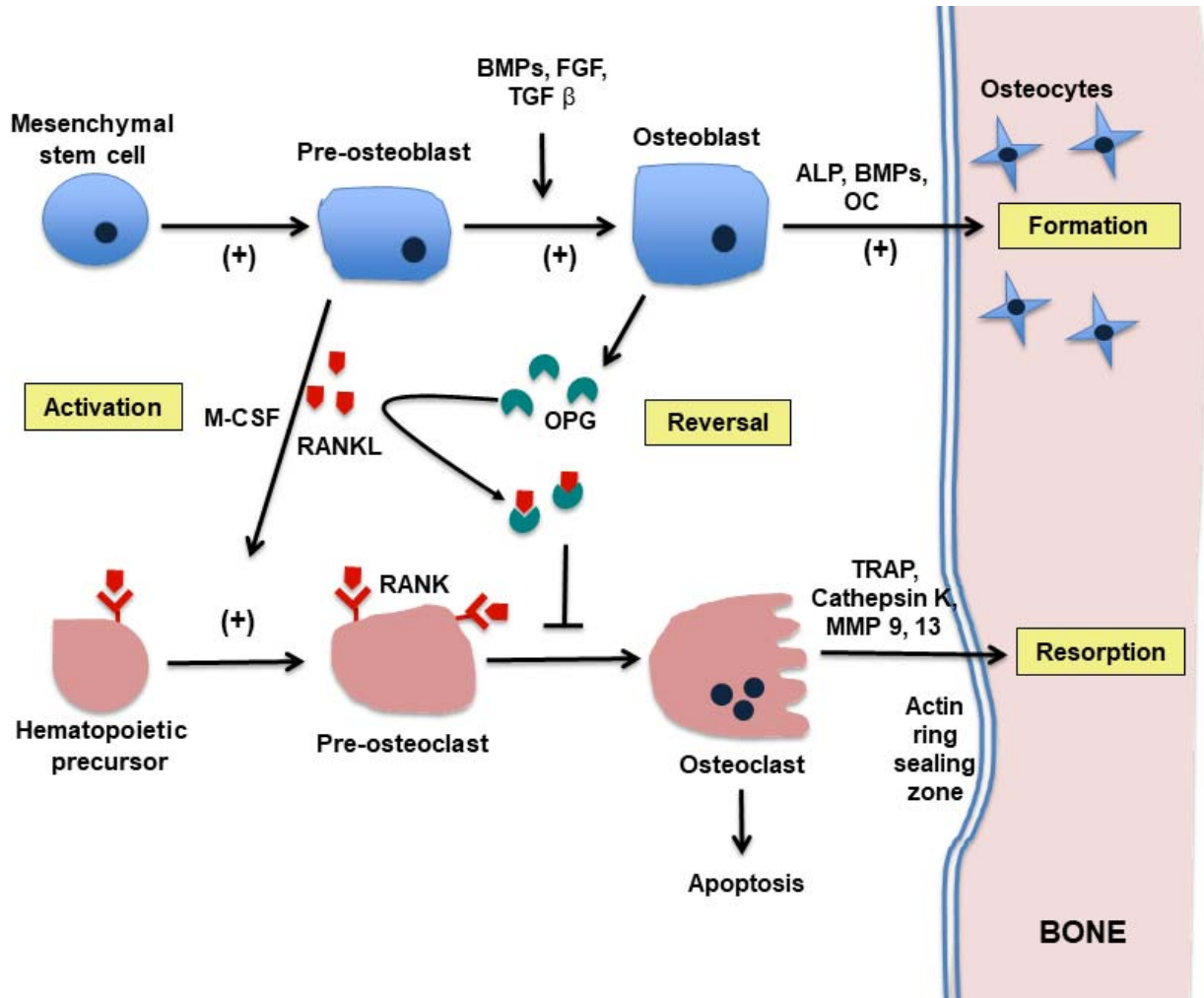
Remodeling starts with the activation of latent bone-lining osteoblast precursors in response to mechanical load shifts. Mesenchymal stem cells and pre-osteoblasts increase their own surface expression of receptor activator of nuclear factor  $\kappa$ B ligand (RANKL) and M-CSF.

RANKL and M-CSF promote the release of immature progenitors of osteoclasts, such as mononuclear monocytes into the circulation and their recruitment to the resorption surface. They also bind to their respective cell surface receptors on pre- osteoclasts [(i.e. RANKL to RANK and M-CSF to colony stimulating factor 1 receptor (CSF1R))] to stimulate the fusion of monocytes into pre-osteoclasts and eventually into multinucleated fully functional osteoclasts. The osteoclastic activation phase lasts for approximately 10 days and precedes bone resorption, reversal and formation (**Figure 1**).

In the resorption phase, osteoclasts attach to the underlying bone matrix and produce actin ring-shaped sealing zones enclosed by a ruffled border, which isolates the bone resorbing compartment from the surrounding extracellular fluid. Osteoclasts then secrete tartrate resistant acid phosphatase (TRAP), cathepsin K and matrix metalloproteinases (MMP) 9 and 13, resulting in the dissolution of inorganic minerals from the bone leading to the degradation of organic matrix. The resorption phase continues for two to three weeks (~21 days) and ends with osteoclast apoptosis.

The five-day reversal phase starts afterwards, which involves cessation of the resorption phase and then a transition towards the osteoblast-mediated formation phase. Osteoclasts stimulate bone formation following resorption by releasing stimulatory paracrine factors embedded in the bone matrix, including insulin like growth factor (IGF) I and II, fibroblast growth factor (FGF), transforming growth factor (TGF) 1 and 2, bone morphogenetic proteins (BMPs) 2, 3, 4, 6 and 7 and platelet-derived growth factor (PDGF). In addition, the osteoblast-osteoclast contact-dependent ephrin signaling pathway, which mediates the interaction between osteoclast-derived ephrinB2 and osteoblast-derived EphB4, suppresses osteoclastogenesis when the two bone cells come into contact with each other to initiate osteoblastogenesis ([Sims and Gooi 2008](#), [Henriksen](#),

[Neutzky-Wulff et al. 2009](#), [Eriksen 2010](#)).



**Figure 1. Different phases of bone remodeling cycle.** Activation, resorption, reversal and formation. RANKL= receptor activator of nuclear factor κB ligand, M-CSF= macrophage colony stimulating factor, CSF1R= colony stimulating factor 1 receptor, OPG= osteoprotegerin, BMP= bone morphogenetic protein, FGF= fibroblast growth factor, TGFβ= transforming growth factor β, ALP= alkaline phosphatase, OC= osteocalcin, TRAP= tartrate resistant acid phosphatase, MMP= matrix metalloproteinase. Figure adapted from Maria *et al*, 2014 ([Maria and Witt-Enderby 2014](#)).

Mature osteoblasts start producing osteoprotegerin (OPG), which acts as a decoy receptor



for RANKL to negatively regulate RANK-RANKL binding and osteoclastogenesis. An inverse correlation exists between OPG and RANKL expression levels, which depends on the state of osteoblast differentiation (i.e., OPG levels are highest when osteoblasts are mature and RANKL levels are highest in immature pre-osteoblasts). Bone formation takes place for four to six months. Here, osteoblasts proliferate and synthesize new bone matrix (osteoid) that serves as a template for mineralization resulting from the accumulation of calcium phosphate hydroxyapatite crystals. Several other factors contribute to osteoblastic bone formation which include alkaline phosphatase (ALP) and osteocalcin (OC). ALP is used as a marker of bone formation because its activity increases with an increase in osteoblast differentiation; however, ALP activity does not always correlate with bone mineralization. OC is another bone formation marker, which increases bone mineralization by regulating the growth of apatite crystals and also plays an important role in fracture resistance ([Gasser and Kneissel 2017](#)). Osteoblasts then undergo one of the following three fates—they either undergo apoptosis, they become entrapped in the mineralized bone matrix as terminally differentiated ‘osteocytes’ or they remain as inactive cells lining the bone surface. These apparently quiescent cells reactivate when new bone formation is required. Primary mineralization accounts for approximately 70% of the mineral deposition that occurs in bone and this process takes two to three weeks. Secondary mineralization, which entails the maturation of mineralization crystals, may require more than a year to accomplish. During high bone turnover, tissue mineralization decreases and the degree of heterogeneity in mineralized matrix increases leading to bone loss. Balanced mineralization is required for optimal bone quality as high degree of mineralization makes new bone more rigid but breakable, whereas low mineralization makes bone less rigid but tough ([Boyce and Xing 2008](#), [Matsuo and Irie 2008](#), [Seeman 2008](#), [Sims and Gooi 2008](#), [Henriksen, Neutzsky-Wulff et al. 2009](#), [Eriksen 2010](#), [Raggatt and Partridge 2010](#),

[Iolascon, Frizzi et al. 2014](#), [Gasser and Kneissel 2017](#)). The mechanisms governing bone remodeling are still not clear. An accurate understanding of the mechanism(s) that couples bone formation and resorption is essential to successfully design interventions for preventing bone loss while upholding bone quality.

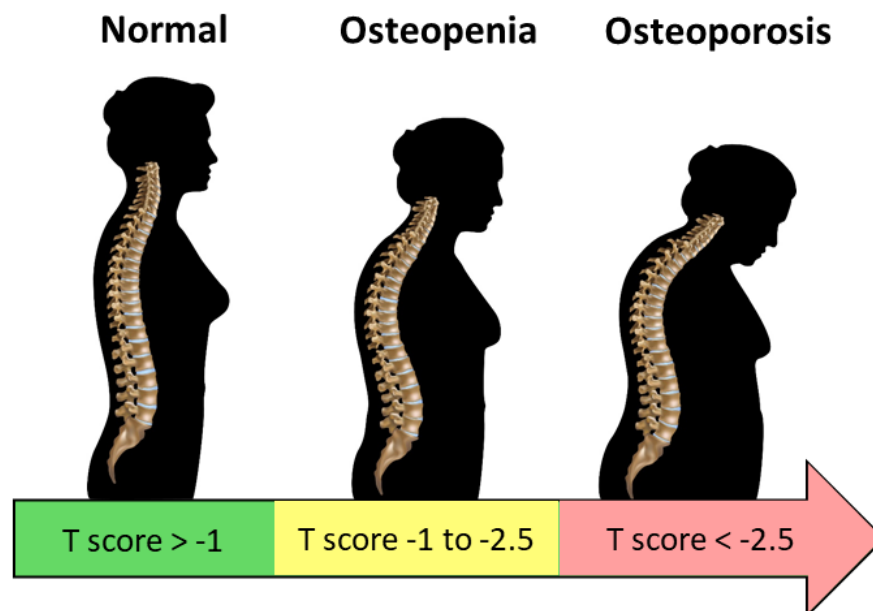
## **1.2 Bone loss- osteopenia and osteoporosis**

### **1.2.1. Introduction to osteopenia and osteoporosis**

Osteopenia and osteoporosis primarily emerge from a disruption in the well-orchestrated and equalized functions of osteoblast and osteoclast and shifting towards greater osteoclastic bone resorption in bone remodeling ([Feng and McDonald 2011](#)). Osteopenia (from Greek ‘*ostoun*’ meaning bone and ‘*penia*’ meaning deficiency), also known as “low bone mass”, is the first stage of bone loss where bone mass begins to decline because of irregular bone remodeling. Osteopenia is characterized as below normal bone density and the precursor to osteoporosis, with a T-score that is between or equal to -1 and -2.5 ([Maria and Witt-Enderby 2014](#)) (**Figure 2**). Bone loss typically progresses insidiously and unnoticed during osteopenia until a fracture occurs and/or osteoporosis develops. If a bone density scan by DXA is performed during the osteopenic stage, it can serve as a baseline bone mineral density (BMD) assessment for diagnosis and future monitoring.

The systemic bone disease osteoporosis (from Greek ‘*ostoun*’ meaning bone and ‘*poros*’ meaning passage or pore) is defined based on World Health Organization (WHO) diagnostic criterion of a T-score less than -2.5. This implies that the bone density is moving towards a worsening of bone density by 2.5 standard deviations from the mean bone density of a 30 years old. Characteristic features of osteopenia-osteoporosis include reduction of bone density, degradation of bone tissue and disturbance of bone microarchitecture. This leads to compromised

bone strength and increased fracture risk following minor or no trauma such as a fall from standing height. A normal bone remodeling cycle causes nearly zero net change in bone mass and strength. High osteoclast activity in imbalanced remodeling leads to low bone mass and high intra-cortical and trabecular porosity, resulting in cortical fragility and loss of trabecular continuity. Defective bone microarchitecture accompanied by poor matrix mineralization weakens the bone's ability to withstand mechanical load below the threshold level. As a result, even typical load produces more stress on bone, predisposing one to osteopenia-osteoporosis and fragility fractures ([Seeman 2002](#), [Seeman 2008](#), [Baron and Hesse 2012](#), [Cosman, De Beur et al. 2014](#), [Siris, Adler et al. 2014](#)). Hip and other osteoporotic fractures, which typically occur at the lumbar spine, femoral neck and wrist, are associated with high morbidity and mortality ([Baron and Hesse 2012](#)).



*Figure 2: Different stages of bone loss*

### 1.2.2. Risk factors for osteopenia and osteoporosis

Osteopenia-osteoporosis can be classified as primary or secondary based on underlying risk factors. Primary bone loss progresses with age and typically results in approximately 10% loss

of bone mineral mass and trabecular bone volume per decade starting after age 40 ([Hui, Slemenda et al. 1990](#)). Although the structural bone damage is similar in men and women during youth, elderly women are often more vulnerable to bone loss than elderly men. This is due, in part, to the fact that men have larger skeletons than women and have thicker trabecular density and so their bones can tolerate more absolute loads. Menopause-related estrogen deficiency is another great contributor to bone loss in elderly women and a rapid decline in bone is observed in the first three years of menopause ([Seeman 2002](#), [Seeman 2008](#), [Macdonald, Nishiyama et al. 2011](#), [Baucom, Pizzorno et al. 2014](#)). Other common risk factors for primary osteopenia-osteoporosis include malnutrition, smoking, sedentary and/or irregular lifestyle, family history, inadequate intake of calcium, and heavy alcohol consumption ([Loh and Shong 2007](#), [Cosman, De Beur et al. 2014](#)). Vitamin D<sub>3</sub> insufficiency has been established in several studies in the last 20 years as yet another significant risk factor of primary osteopenia-osteoporosis ([Baucom, Pizzorno et al. 2014](#)). Secondary osteopenia-osteoporosis mostly emerges from prolonged use of glucocorticoid therapy ([Angeli, Guglielmi et al. 2006](#)), or from diseases such as thyroid disorders, rheumatoid arthritis, diabetes mellitus ([Inaba 2004](#)), and numerous hematological, gastrointestinal, neurological and mobility disorders ([Loh and Shong 2007](#), [Mohammad, Khan et al. 2009](#), [Cheng, Lin et al. 2014](#), [Cosman, De Beur et al. 2014](#)). Psychological conditions such as depression as well as poor sleep also contribute to bone loss, osteopenia and osteoporosis and related fractures ([Brassington, King et al. 2000](#), [Cizza, Primma et al. 2009](#)). Finally, recent studies have shown that chronic circadian, sleep and melatonin disruption (e.g. in shift workers) can adversely affect bone resulting in low bone density and increased risk of hip and wrist fractures ([Quevedo and Zuniga 2010](#), [Kim, Choi et al. 2013](#), [St Hilaire, Rahman et al. 2018](#)).

### 1.2.3. Prevalence of osteopenia and osteoporosis

Osteopenia and osteoporosis are among the most common skeletal disorders worldwide, taking a toll on our world's overall health status. The International Osteoporotic Foundation (IOF) has reported ~ 200 million sufferers (age > 50) of osteoporosis worldwide ([Reginster and Burlet 2006](#)). Based on a recent survey by the National Osteoporosis Foundation (NOF), it is estimated that 10.2 million US adults suffer from osteoporosis at present and another 43.4 million osteopenic population are under great threat of developing osteoporosis in the near future. This accounts for 54.2% of the total US adult population with bone loss irrespective of ethnicity ([Wright, Looker et al. 2014](#)). These numbers are predicted to increase up to 11.9 million for osteoporosis and 64.3 million for osteopenia by 2030 ([Maria and Witt-Enderby 2014](#)). The European Union also shows a similar prevalence with an estimated 22 million women and 5.5 million men (50-84 years) with bone loss and, if left untreated, these numbers will rise to 23% by 2030 ([Hernlund, Svedbom et al. 2013](#)). For the rest of the world, bone loss scenarios are similar. For example, in Asia a high prevalence for osteopenia and osteoporosis exists and these are conservative estimates considering that bone loss is highly undiagnosed in most Asian countries until a fracture occurs and, even if this happens, many fractures are treated conservatively at home rather than in hospitals ([Siris, Adler et al. 2014](#)).

The increased prevalence of bone loss is also rapidly increasing the risk of its life-threatening consequence— fractures. Nearly 8.9 million annual fractures are reported worldwide, accounting for one fracture every three seconds. Fracture rate is higher in women than men with one in three women and one in five men over age 50 experiencing a fracture. Overall 61% of osteoporotic fractures occur in women and most occur in the humerus, hip and spine ([Johnell and Kanis 2006](#), [Sale, Beaton et al. 2014](#)). In the US, the annual fracture rate is 1.5 to 2 million per

year ([Burge, Dawson-Hughes et al. 2007](#)) and the rate is expected to increase to 3 million by 2025. Among all fractures, hip fractures are responsible for 8.4 to 36% mortality within a year of incidence in the US. Only 40% of the population with hip fractures fully recover to their pre-fracture health following long-term care. Even though vertebral fractures are clinically silent at the initial phase, they are the chief predictor of future fracture risk. For example, if one experiences a vertebral fracture, then their risk for a subsequent vertebral fracture increases 5-fold. For a non-vertebral fracture, the risk increases 2- to 3-fold ([Cosman, De Beur et al. 2014](#)). Twice the number of fractures arise in women with osteopenia. This is significant considering that women with osteopenia represent almost 50% of the total population at risk ([Pasco, Seeman et al. 2006](#)) and the prevalence of osteopenia is 3.4 times more than osteoporosis ([Pfister, Welch et al. 2016](#)).

Bone-related disorders also contribute to the global health burden in an indirect way through back pain, height loss, spine deformity and disability ([Cosman, De Beur et al. 2014](#)). Fracture is often accompanied by long-term chronic pain, greater disability, poor quality of life (QOL) and early death. On a day-to-day basis, osteoporosis affects one's physical state of health such as chronic daily backache, a limited social life and loss of free movement ([Brenneman, Barrett-Connor et al. 2006](#), [Masaryková, Fulmeková et al. 2015](#)). Besides these, morbidity and mortality rates associated with osteoporotic fractures are increasing and posing huge economic and social burdens. For example, the annual fracture-related treatment and post-treatment care expense is \$19 billion in the US, and €37 billion in the European Union ([Johnell and Kanis 2006](#), [Burge, Dawson-Hughes et al. 2007](#), [Kanis, Odén et al. 2012](#), [Hernlund, Svedbom et al. 2013](#), [Maria and Witt-Enderby 2014](#)). The projected annual total fracture cost across all fracture types for US women is more than 18 billion by 2025, which is more than that of myocardial infarction, stroke and breast cancer ([Singer, Exuzides et al. 2015](#)).

### 1.3. Menopause and bone loss

The menopausal transition is characterized by irregular lengths of the menstrual cycle and natural amenorrhea (absence of menstrual period) for at least one year accompanied by elevated serum levels of follicle-stimulating hormone (FSH). Postmenopause refers to the period where menstruation ceases permanently due to a complete loss of follicular activity in the ovary. Despite being a natural physiological phenomenon, the menopausal transition is often accompanied by debilitating consequences of osteopenia, osteoporosis and related fractures ([Greendale, Sowers et al. 2012](#), [Finkelstein, Brockwell et al. 2013](#)). In addition to age and gender, hormonal status affects the steady diurnal rhythm of bone remodeling ([Maria and Witt-Enderby 2014](#)). Low estrogen levels as observed in postmenopause increases the life span of osteoclasts and stimulates loss of connectivity between trabecular bone units. Increases in trabecular porosity leads to attenuated bone strength, specifically in the vertebrae; this results in more vertebral fractures in women than men ([Seeman 2002](#), [Seeman 2008](#)).

Women begin to experience bone loss during late perimenopause, which continues even after menopause. The annual rate of bone loss is typically slow and steady with a rate of  $\sim 0.4\%$ , which increases to 2 to 5% per year for the first 5 to 10 years following menopause ([Riis, Hansen et al. 1996](#)). Total trabecular and cortical bone loss in women during the course of their lifetime is about 50% and 30%, respectively, half of which occurs in the first 10 years of menopause ([Finkelstein, Brockwell et al. 2013](#)). After the first 15 years of menopause, cortical bone loss surpasses trabecular bone loss, resulting in fragility fractures ([Zebaze, Ghasem-Zadeh et al. 2010](#)). Bone biopsies of normal postmenopausal women show an increase in the number of resorption pits on the bone surface indicating an increase in bone resorption ([Garnero, Sornay-Rendu et al. 1996](#)).

Currently, about 30% of postmenopausal women in the western world are suffering from osteoporosis and 40% of them have high risk of having one or more fractures in their lifetime ([Reginster and Burlet 2006](#)). The US Preventive Services Task Forces recommends that all women aged 65 years or older should undergo regular BMD screening. The International Society for Clinical Densitometry recommends an earlier screening for women (age <65 years), who are at high risk of developing fracture due to low body weight, prior fracture history or high-risk medication intake; the screening age for men is usually 70 years or above ([Lim, Hoeksema et al. 2009](#)). Interestingly, a study involving 149,524 white postmenopausal women (mean age 64.5 years) estimates that 82% of new fractures occurs in women with low bone mass (osteopenia) after one year ([Siris, Chen et al. 2004](#)). Similar outcomes were observed in a 5.6-year study with postmenopausal women ([Pasco, Seeman et al. 2006](#)), suggesting that although osteoporosis is a high risk stage for recurrent fractures in postmenopausal women, initial fractures mostly arise in this cohort when they are osteopenic. All these factors necessitate the need of proper attention and care for the osteopenic population as well as the osteoporotic population to minimize fracture-related morbidity and mortality.

In addition to bone loss, more than 80% postmenopausal women suffer from numerous physical and psychological symptoms with varying degrees of severity that disrupt their life. Major menopause-related physical symptoms include hot flashes, night sweats, fatigue, sleep disturbances, breast pain, palpitations, weight gain, urinary incontinence and vaginal dryness. Psychological health disturbances result in the emergence of anxiety, stress and depression. In fact, studies show that women experience at least one or more of these symptoms while transitioning through menopause. The large multiethnic Study of Women's Health Across the Nation (SWAN) explained that all these changes collectively have a debilitating effect on the overall health-related



quality of life in this cohort. Health-related quality of life (QOL) is a part of the broad, multidimensional concept “Quality of life” that specifically measures how an individual’s life is affected by changes in his/her health status based on following aspects: physical health and performance, emotional activity, role limitations, and social functioning. The impact of menopause on health-related QOL is an important outcome measure in health sectors to provide effective treatment and care to the symptomatic postmenopausal women ([Avis, Colvin et al. 2009](#), [Greenblum, Rowe et al. 2013](#)).

#### **1.4. Bone loss therapy—current and future perspectives**

Most of the conventional pharmaceutical bone loss therapies are treatment-focused rather than preventative, focusing primarily on attenuating further bone loss by inhibiting osteoclast function. Although these antiresorptive therapies lower fracture rate by 30 to 50%, they do not result in new bone formation or cortical microarchitecture improvement, which is essential during late-stage osteoporosis to maintain bone health and prevent fragility fractures. Even though clinical trials show increases in BMD with antiresorptives, this occurs by the secondary mineralization of existing (but declined) bone tissue rather than through the formation of new healthy bone. For instance, bisphosphonates (e.g. alendronate), which are the most effective first line bone loss therapies prevent degeneration of cancellous bone and thinning of cortex by inhibiting bone resorption; however, alendronate does not improve bone mass and microarchitecture. Bone anabolism needs to be an integral part of new bone loss therapies to aid in restoring bone mass and microarchitecture to protect mechanical integrity. A lack of bone-tropic properties in current antiresorptives makes them less than ideal for improving bone quality, density and bone strength ([Boivin and Meunier 2002](#), [Lyritis, Georgoulas et al. 2010](#), [Iolascon, Frizzi et al. 2014](#)).

Despite the ample availability of conventional osteoporosis drugs, an alarming rise in the

prevalence of osteopenia-osteoporosis as well as the rate of fracture-related morbidity and mortality are still being observed in the geriatric population ([Reginster and Burlet 2006](#)). The reasons for this are not clear but probably associated with their adverse effects in the body and due to a lack of compliance ([Caro, Ishak et al. 2004](#)). Bisphosphates remain in the bone remodeling area for years even after discontinuation of the therapy due to their high affinity for calcium. This long residence time in bone contributes to the occurrence of osteonecrosis of the jaw, atypical femoral shaft fractures and hypocalcemia. The concept of a “three to five years bisphosphonate drug holiday” has emerged to avoid these circumstances. Discontinuation of bisphosphonate therapy, however, will halt these adverse effects, but will not prevent further bone loss and may, in fact, accelerate bone loss ([Watts and Diab 2010](#)). Denosumab, a monoclonal RANKL inhibitor, has been found to produce eczema and cellulitis in the FREEDOM (Fracture Reduction Evaluation of Denosumab in Osteoporosis Every 6 Months) and HALT (Hormone Ablation Bone Loss Trial) clinical trials, as well as osteonecrosis of the jaw and atypical femur fractures, result from its blood calcium lowering effects ([Scotland, Waugh et al. 2011](#), [Diz, López-Cedrún et al. 2012](#)). Hormone therapy (HT) such as selective estrogen receptor modulators (SERMs) are popular among women going through the menopausal transition due to their positive effects on menopausal symptoms (e.g., vasomotor symptoms). However, awareness has risen towards the serious side-effects such as mammary cancer, deep venous thrombosis and other cardiovascular events associated with long-term use of HT ([Grady, Ettinger et al. 2004](#), [Baucom, Pizzorno et al. 2014](#)). The only anabolic therapy available in the market is teriparatide— a parathyroid hormone (PTH) analog. Because long-term teriparatide treatment increases the incidence of rat osteosarcoma, its use is FDA-restricted to two years ([Vahle, Sato et al. 2002](#)). These factors contribute to limited adherence and poor compliance amongst those with diagnosed osteoporosis to the current antiresorptive

therapies. Particularly in postmenopausal women, where the relationship between compliance and adherence to the therapy was measured by their medication possession ratio, low compliance caused a 17% increase in fracture rate, a 37% increase in the risk of hospitalization and associated cost of medical services. Bisphosphonate studies show that among the osteoporotic women aged 45 years or older, only 50% new users were compliant after first three months, which dropped to 30% after one year and 16% after three years ([Warriner and Curtis 2009](#), [Silverman and Gold 2010](#), [Genuis and Bouchard 2012](#)).

For those individuals with osteopenia, the pharmacological treatment regimen is typically delayed or given “watchful waiting” until the transition to osteoporosis is diagnosed (T-score less than -2.5 at the femoral neck/spine). Interventions may also occur if the individual with osteopenia has a history of having a previous hip or vertebral fracture; or when the T score is between -1.0 and -2.5 at the femoral neck/spine and the 10-year risk of hip fracture  $\geq 3\%$ ; or the 10-year risk of major osteoporosis-related fracture  $\geq 20\%$  by Fracture Risk Assessment Tool (FRAX<sup>®</sup>) calculation ([Watts, Lewiecki et al. 2008](#)). The osteopenic phase of bone loss consists mostly of unawareness, no treatment or a non-pharmacological self-care approach through diet, exercise and micronutrient supplementation. The non-pharmacologic treatment guidelines for osteopenia and osteoporosis routinely recommend calcium and vitamin D supplementation as a prevention; however, neither alone has proven to have any significant effect for reducing the incidence of fractures or preventing osteoporosis; and taking the combination of calcium plus D only slightly reduces the risk of hip and other fractures ([Avenell, Mak et al. 2014](#), [Bolland, Leung et al. 2015](#)). A diagnosis of osteopenia is important considering the fact that a majority of osteopenic individuals will develop osteoporosis within 10 years ([Kanis and Organization 2008](#)). Osteopenia often carries a significant treatment uncertainty during the time of the greatest fracture risk burden ([Pfister, Welch et al.](#)

[2016](#)). Therefore, the current treatment approach to osteopenia as compared to osteoporosis could be analogous to the progression and treatment options of prediabetes as compared to diabetes, considering the similar risk factors between two phases of each disease. Interventions at this stage focusing on bone anabolism would be a critical first step for maintaining normal bone integrity and to prevent future fractures ([Lyritis, Georgoulas et al. 2010](#)). Thus, novel and safe bone loss therapies targeting the function of both osteoclasts and osteoblasts to minimize bone loss and advance bone growth without producing adverse effects is warranted.

### **1.5. Research objective**

The “silent” disease osteopenia and subsequent osteoporosis are creating a loud impact in terms of morbidity, mortality and greater economic burden in the life of postmenopausal women. In addition to bone loss, their transitioning through several menopausal and psychological changes as well as the side effects and treatment costs associated with current therapies tallying to their suffering and thus worsening their health-related QOL and reducing compliance rate. Therefore, an ideal bone therapy that is efficacious with high compliance for postmenopausal cohort should satisfy both the objective health outcome (e.g., improves bone growth) and subjective health outcome (e.g., improves compliance). These observations lend support for the development of a safe alternative to current therapies to ensure maximum bone health improvement with minimum side effects.

The objective of this study entitled Melatonin-micronutrients Osteopenia Treatment Study (MOTS) was to develop a safe and effective bone loss therapy for the osteopenic population. Interventions given at this stage of bone loss are expected to not only stop bone loss but also enrich new bone growth and improve overall QOL to prevent its progression to osteoporosis and fractures. In pursuit of study goal, a unique combination of natural bone tropic agents— melatonin,

strontium citrate, vitamin K<sub>2</sub> and vitamin D<sub>3</sub> was developed and referred to as “MSDK”. The MOTS investigated if MSDK could improve bone density and bone turnover and health-related QOL in postmenopausal women and if this was due to MSDK-mediated modulation of the bone remodeling process.

## **1.6. Effects of melatonin, strontium citrate, vitamin D<sub>3</sub> and vitamin K<sub>2</sub> on bone**

### **1.6.1. Melatonin**

Melatonin, the first compound of interest in this therapy, is the endogenous chronobiotic molecule that is synthesized and secreted during the hours of darkness from the pineal gland. Melatonin is well known for its effects on sleep; however, supplementation with melatonin also produces favorable effects on bone including increasing bone density and reversing bone loss in models of osteoporosis as reviewed ([Maria and Witt-Enderby 2014](#)). Melatonin synchronizes circadian rhythms in bone metabolism, in part, and favors bone growth when present at a higher level ([Witt-Enderby, Radio et al. 2006](#), [Maria and Witt-Enderby 2014](#)). This is yet another important mechanism of melatonin’s considering that decreased nocturnal melatonin levels due to aging, light exposure at night or poor sleep quality increases the risk of bone loss and related fractures ([Sandyk and Awerbuch 1992](#), [Cardinali, Ladizesky et al. 2003](#), [Ostrowska, Kos-Kudla et al. 2003](#), [Feskanich, Hankinson et al. 2009](#), [Witt-Enderby, Slater et al. 2012](#), [Maria and Witt-Enderby 2017](#)). Melatonin has been shown clinically in the Melatonin Osteoporosis Prevention Study (MOPS; NCT01152580) to renormalize osteoclast:osteoblast ratios (NTx: Osteocalcin) back to equilibrium and improve the physical symptoms of menopause in healthy perimenopausal women; this occurred following a nightly intake of 3 mg melatonin for six months ([Kotlarezyk, Lassila et al. 2012](#)). The MOPS was a double-blind randomized placebo-controlled trial (RCT) involving 18 perimenopausal women (ages 45-54) with a T-score > -2.0. In a follow-up study of

the MOPS using a therapeutically equivalent concentration of melatonin, it was shown that melatonin (50nM) given for 21 days to human mesenchymal stem cells (hMSCs) and peripheral blood monocytes (hPBMCs) grown as co-cultures induces osteoblast differentiation. The effect of melatonin on osteoclast differentiation is dependent upon the type of co-culture—layered or transwell where melatonin inhibits osteoclast differentiation in the layered co-culture (when hMSCs and hPBMCs were in contact with one another) and is without effect in the transwell co-culture (when hMSCs communicated with hPBMCs via paracrine effects) ([Maria, Samsonraj et al. 2017](#)). The Treatment of Osteopenia with Melatonin study (MelaOst), which was another double-blind placebo-controlled RCT involving 81 postmenopausal women with osteopenia (ages 56-73), demonstrates that one-year nightly dosing with melatonin (1mg, 3mg) increases femoral neck BMD compared to placebo. This study also demonstrates that urinary calcium excretion decreases in women taking melatonin as compared to those taking placebo suggesting that the bone density increases were due, in part, to melatonin-mediated increases in bone mineralization. Melatonin is shown to benefit metabolic conditions in this cohort as a decrease in total fat mass and a trend towards an increase in lean body mass occurs in women taking melatonin but not placebo ([Amstrup, Sikjaer et al. 2015](#), [Amstrup, Sikjaer et al. 2015](#)). This important clinical finding served as the rationale as to why the metabolic proteins, peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), glucose transporter type 4 (GLUT4) and beta subunit of insulin receptor (IR $\beta$ ), were assessed in the co-culture model because melatonin may induce osteoblastogenesis by regulating the fate of the mesenchymal stem cell from adipogenesis to osteogenesis as recently reported ([Maria, Samsonraj et al. 2017](#)).

The outcomes from the two clinical studies, MOPS and MelaOst, are supported by myriad preclinical and *in vitro* models, which revealed melatonin's inducing effects on osteoblastogenesis;

inhibitory effects on osteoclastogenesis; and inducing effects on bone formation ([Maria and Witt-Enderby 2014](#)). Melatonin promotes osteoblastogenesis by enhancing the differentiation of mesenchymal stem cells (hMSCs) and pre-osteoblasts into osteoblasts ([Radio, Doctor et al. 2006](#), [Zaminy, Ragerdi Kashani et al. 2008](#), [Sethi, Radio et al. 2010](#), [Zhang, Su et al. 2010](#), [Park, Kang et al. 2011](#)). Melatonin stimulates osteoblast proliferation ([Nakade, Koyama et al. 1999](#), [Satomura, Tobiume et al. 2007](#)); and suppresses osteoclastogenesis and bone resorption ([Koyama, Nakade et al. 2002](#), [Suzuki and Hattori 2002](#)) *in vitro*. These data correlate with *in vivo* findings from our lab where exogenous melatonin intake for one year increases bone density in an intact female mouse model similar to that induced by HT ([Witt-Enderby, Slater et al. 2012](#)). Melatonin linked to calcium aluminate scaffolds also induces bone regeneration in ovariectomized (estrogen-depleted) female rats ([Clafshenkel, Rutkowski et al. 2012](#)) and augments the bone-forming effects of estradiol in ovariectomized female rats ([Ladizesky, Boggio et al. 2003](#)). Because these models mimic the postmenopausal condition where levels of both estrogen and melatonin decline suggests that restoration of nocturnal melatonin peaks with exogenous melatonin may help to offset the bone loss that also occurs during this time in a woman's life as reviewed ([Witt-Enderby, Radio et al. 2006](#)).

Besides producing direct effects on bone density, melatonin shows favorable effects on the physical symptoms of menopause (such as vasomotor symptoms and bloating) in the MOPS cohort ([Kotlarczyk, Lassila et al. 2012](#)). Further assessments of the MOPS daily diary logs also reveal that melatonin improves subject-reported mood and sleep quality in this cohort ([Maria, Samsonraj et al. 2017](#)) and also improves depression, anxiety and stress ([Bellipanni, Bianchi et al. 2001](#), [Haridas, Kumar et al. 2013](#), [Hansen, Andersen et al. 2014](#), [Sun, Wang et al. 2017](#), [Zhang, Guo et al. 2017](#)). Psychological health plays a prominent role in maintaining bone health. For example,

depression is found to significantly correlate with lower BMD in the lumbar spine and femur and high cortisol levels in postmenopausal women ([Furlan, Ten Have et al. 2005](#)). Sleep deficiency due to shift work ([Feskanich, Hankinson et al. 2009](#), [Quevedo and Zuniga 2010](#), [Kim, Choi et al. 2013](#), [Wang, Wu et al. 2015](#)) or stress produces adverse effects on bone and these may be attributed to their effects on melatonin levels; because shift work and stress have been shown to decrease melatonin levels as reviewed ([Maria and Witt-Enderby 2017](#)). All these findings coupled with its relative safety in aging population ([Witt-Enderby, Radio et al. 2006](#), [Maria and Witt-Enderby 2014](#)) makes melatonin an important component of MSDK to improve not only bone health, but also QOL and compliance associated with it.

### **1.6.2. Strontium**

Strontium is the 15<sup>th</sup> most abundant alkaline earth metal named after the Scottish village Strontian where it was first discovered ([Murray 1993](#)). The adult body burden of strontium is about 0.3–0.4 g, with 99% found in the skeleton as bone or teeth ([Cabrera, Schrooten et al. 1999](#)). Strontium is available as ranelate, citrate, chloride, carbonate or lactate. Among them, strontium ranelate is an approved therapy for osteoporosis treatment in Europe and Australia. In the US, strontium citrate is the FDA-approved bone support supplement. It is available over-the-counter with a typical 680mg recommended dosage as compared to the 2g therapeutic dose for strontium ranelate ([Hernlund, Svedbom et al. 2013](#)).

Numerous clinical studies have shown beneficial effects of elemental strontium on bone health in the postmenopausal osteopenic and osteoporotic population, specifically on vertebral and femoral bone density and vertebral and non-vertebral fracture risk ([Meunier, Roux et al. 2004](#), [Reginster, Seeman et al. 2005](#), [Malaise, Bruyere et al. 2007](#)). The therapeutic efficacy of strontium ranelate in postmenopausal osteoporosis was established following two major clinical trials:



Treatment of Peripheral Osteoporosis (TROPOS) and Spinal Osteoporosis Therapeutic Intervention (SOTI), respectively. SOTI was a phase III double-blind placebo-controlled RCT, which included 1649 postmenopausal women (mean age 70) with osteoporosis and at least one vertebral fracture. This cohort received 2gm of strontium ranelate or placebo orally daily for one to three years. In this study, strontium ranelate increases lumbar spine and femoral neck BMD by 12.7% and 7.2%, respectively, after one year; which increases up to 14.4% and 8.3%, respectively, after three years compared to placebo ([Meunier, Roux et al. 2004](#)). TROPOS was another double-blind placebo-controlled RCT involving 5091 postmenopausal women with osteoporosis (age >70). In this study, strontium ranelate (2gm/day) increases femoral neck and total hip BMD by 8.2% and 9.8%, respectively after three years vs. placebo ([Reginster, Seeman et al. 2005](#)). A post-hoc analysis performed on 1428 postmenopausal osteopenic women chosen from the SOTI and TROPOS cohort (based on having osteopenia at a non-osteoporotic site at baseline) reveals that strontium ranelate renormalizes spine and hip BMD at a progressive level after one, two and three years of treatment and is well-tolerated in this population ([Malaise, Bruyere et al. 2007](#)). Strontium incorporates mostly in newly formed mineralized bone structures without replacing calcium from the existing bones; this is to maintain secondary mineralization at a normal level. This is supported by the analysis of bone biopsies in postmenopausal women treated chronically with strontium ranelate, which demonstrates that strontium preserves bone microarchitecture and quality by preserving bone mineralization (density and heterogeneity at tissue level). These findings further support a role for strontium at reducing fracture risk ([Ammann, Badoud et al. 2007](#), [Li, Paris et al. 2010](#), [Doublier, Farlay et al. 2011](#)).

Despite being available as an over-the-counter supplement for bone health, the underlying mechanism of strontium citrate on bone health has yet to be determined. To our knowledge, the

MOTS is one of the first trials to evaluate the efficacy and safety of strontium citrate in treating postmenopausal osteopenia when used in a combination with melatonin, vitamin D<sub>3</sub> and vitamin K<sub>2</sub>. Out of the very few studies available on strontium citrate, one significant study worth mentioning is the Combination of Micronutrients for Bone (COMB) study. The COMB assessed the efficacy of a one-year treatment with strontium citrate (680mg), vitamin D<sub>3</sub> (2000IU) and vitamin K<sub>2</sub> (100mcg) on bone density in 77 participants; 72 of the 77 were females of which 56 were postmenopausal and 5 of the 77 were males ([Genuis and Bouchard 2012](#)). Although the citrate form slightly differs from the ranelate in terms of their chemical structure, the efficacy of strontium citrate in improving BMD is equal to that of strontium ranelate ([Genuis and Bouchard 2012](#)), probably because strontium is equally delivered to bone regardless of the analogues ([Wohl, Chettle et al. 2013](#)). However, strontium citrate is associated with less side effects ([Genuis and Bouchard 2012](#)).

*In vitro* and preclinical studies have also supported the clinical data by demonstrating that strontium maintains skeletal morphology and bone remodeling via multiple mechanisms (**Figure 3**). Strontium stimulates osteoblast growth, maturation, function and survival ([Atkins, Welldon et al. 2009](#), [Fromigué, Haÿ et al. 2009](#), [Peng, Zhou et al. 2009](#), [Saidak, Haÿ et al. 2012](#), [Querido and Farina 2013](#), [Almeida, Nani et al. 2016](#)) and inhibits osteoclastogenesis ([Bonnellye, Chabadel et al. 2008](#), [Yamaguchi and Weitzmann 2012](#)). Strontium induces vertebral bone mass in intact female mice with no deleterious effect on bone mineralization ([Delannoy, Bazot et al. 2002](#)). It attenuates loss of trabecular bone, conserves bone strength and microarchitecture while facilitating the fracture healing process in ovariectomized rats ([Bain, Jerome et al. 2009](#), [Thormann, Ray et al. 2013](#)).

### 1.6.3. Vitamin D<sub>3</sub>

Vitamin D, also known as the “sunshine vitamin”, has been recognized as an essential nutrient for bone health since 1930 ([Wacker and Holick 2013](#)). Adequate levels of vitamin D<sub>3</sub> (cholecalciferol) are critical for increasing the intestinal absorption of calcium from 10% to 30-40% ([Kidd 2010](#)). Also, an attenuation of bone loss in the hip is observed with a minimum daily dose of 1000 IU vitamin D<sub>3</sub> in postmenopausal women ([Macdonald, Wood et al. 2013](#)). Apart from vitamin D<sub>3</sub>'s action on the calcium absorption, it performs a dual mode of action on bone favoring both osteogenesis ([Anderson, Lam et al. 2013](#), [Chen, Dosier et al. 2013](#), [Yang, Atkins et al. 2013](#)) and bone resorption ([Bar-Shavit, Teitelbaum et al. 1983](#), [Kitazawa, Kajimoto et al. 2003](#), [Kogawa, Findlay et al. 2010](#), [Anderson, Lam et al. 2013](#)) (**Figure 3**)

### 1.6.4. Vitamin K<sub>2</sub>

Vitamin K<sub>2</sub> (menaquinone 7; MK7) is the approved dietary supplement for osteoporosis in Japan for its efficacy in carboxylating osteocalcin, a crucial biomarker of bone matrix formation. Carboxylation of osteocalcin decreases osteoclast activity and so, during the bone remodeling process, carboxylated osteocalcin will remain in the bone under bone resorption is required where it will then be decarboxylated to activate osteoclasts ([Lacombe and Ferron 2015](#)). A majority of the observational studies found correlations between low vitamin K<sub>2</sub> intake, low bone mass and increases in the risk of fracture ([Frandsen and Gordeladze 2017](#)). In healthy postmenopausal women, vitamin K<sub>2</sub> (MK7) intake at a dose of 180 mcg/day for three years significantly induces serum levels of vitamin K and attenuates age-related decreases in lumbar spine and femoral neck BMD. Vitamin K<sub>2</sub> supplementation also improves bone strength and prevents loss of vertebral height at the lower thoracic region of vertebrae ([Knapen, Drummen et al. 2013](#)). Other studies in postmenopausal women demonstrate that vitamin K<sub>2</sub> (MK7, 375mcg per day for one year) reverses

osteopenia and improves bone microarchitecture ([Rønn, Harsløf et al. 2016](#)). The mechanisms underlying vitamin K<sub>2</sub>'s effects on bone appear to target bone microarchitecture and bone tissue quality by improving cortical thickness, trabecular number and connectivity ([Ammann, Badoud et al. 2007](#), [Iolascon, Frizzi et al. 2014](#)) and by increasing collagen production ([Sato 2014](#)). *In vitro* studies demonstrate stimulatory effects of MK7 on osteoblastogenesis and suppressive effects on osteoclastogenesis ([Yamaguchi, Sugimoto et al. 2001](#), [Katsuyama, Otsuki et al. 2005](#), [Atkins, Welldon et al. 2009](#), [Yamaguchi and Weitzmann 2011](#)) (**Figure 3**).

Although, the MK7 analogue of vitamin K<sub>2</sub> is relatively less studied than the MK4 (menaquinone 4) analogue, the MOTS used MK7 due to its longer half-life in the circulation, its better bioavailability than MK4 and its relative safety when used as a dietary supplement ([Sato 2014](#), [Marles, Roe et al. 2017](#)). This supports our contention that a lower dose of MK7 (60 mcg) will lead to greater K<sub>2</sub> accumulation in extrahepatic tissues compared to MK4 where higher doses would be required to achieve the same effect ([Sato, Schurgers et al. 2012](#)).

### **1.7. Rationale for choosing melatonin, strontium citrate, vitamin D<sub>3</sub> and K<sub>2</sub> (MSDK)**

Combination therapies have demonstrated a greater benefit to preserving bone versus monotherapies since each drug targets a different mechanism (**Figure 3**). Clinically, this is also supported. For example, combination of vitamin D<sub>3</sub> and K<sub>2</sub> increases vertebral bone density in postmenopausal women with osteopenia and osteoporosis compared to each alone ([Ushiroyama, Ikeda et al. 2002](#)). Also, a recent study demonstrates that severe deficiency in vitamins D<sub>3</sub> and K<sub>2</sub> are associated with femoral fracture; these findings imply that adequate supplementation with both of these vitamins is essential when treating patients with osteopenia and osteoporosis ([Franke 2017](#)). Improved vitamin D<sub>3</sub> status in postmenopausal women also significantly enhances BMD in response to strontium ranelate, particularly in the femoral neck ([Catalano, Morabito et al. 2015](#)).

The effects of elemental strontium as well as other micronutrients (vitamin D<sub>3</sub> and K<sub>2</sub>) on bone health in postmenopausal cohort are more pronounced when used in combination. This is demonstrated in the COMB study where a majority (81%) of the postmenopausal cohort with osteopenia or osteoporosis show an increase in BMD in the femoral neck (4%), total hip (3%) and spine (6%). In fact, this supplementation is found as effective as bisphosphonates and strontium ranelate at increasing BMD in a postmenopausal population ([Genuis and Bouchard 2012](#)).

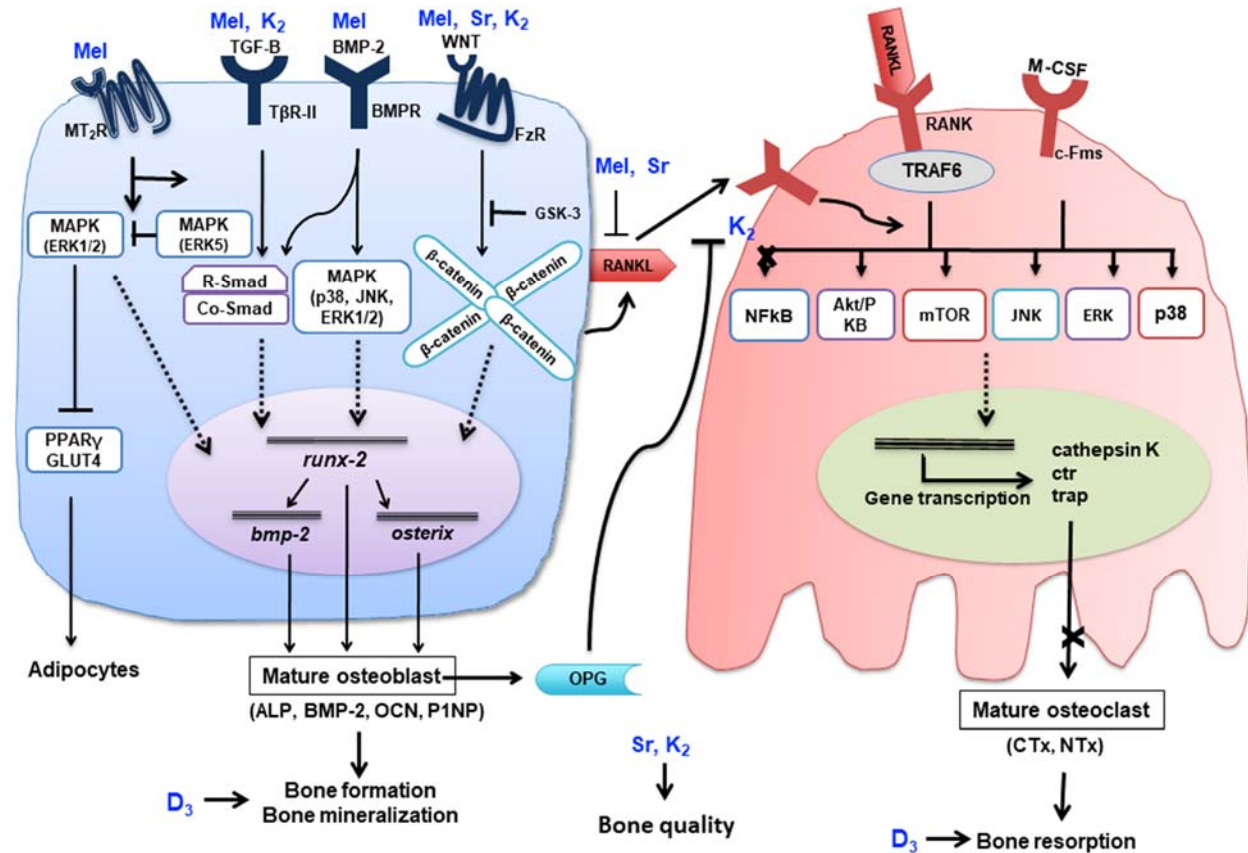
Although the micronutrients used in the COMB study successfully improves bone density, 37 out of 114 participants (32.45%) either were non-compliant or did not complete the intervention ([Genuis and Bouchard 2012](#)). Compliance is a critical factor in developing long-term therapies as low compliance limits adherence and severely hampers the effectiveness of the therapy. Unique to the MOTS was the inclusion of melatonin, which is known for its soporific effects and positive effects on menopause-related physical symptoms, mood and sleep quality ([Kotlarczyk, Lassila et al. 2012](#), [Maria, Samsonraj et al. 2017](#)). In the MelaOst trial, melatonin increases BMD, decreases total fat mass and shows trend towards an improvement in sleep especially in those with poor sleep as measured by a Pittsburgh Sleep Quality Index (PSQI) score greater than 5 ([Amstrup, Sikjaer et al. 2015](#), [Amstrup, Sikjaer et al. 2015](#), [Amstrup, Sikjaer et al. 2015](#)). Because melatonin improves both objective and subjective measures—all of which could improve bone health—melatonin was added in combination with a lower dose of strontium citrate (450mg), vitamin K<sub>2</sub> (60 mcg) and vitamin D<sub>3</sub> (2000 IU) to enhance bone density and improve compliance. Melatonin was also given at night to supplement the nocturnal surge in melatonin which was based on past studies demonstrating that factors that inhibit melatonin production at night, for example, light exposure at night, pinealectomy ([Ostrowska, Kos-Kudla et al. 2003](#)) and shift work ([Feskanich, Hankinson](#)

[et al. 2009](#)) reduce BMD and increase the risk of fractures ([Feskanich, Hankinson et al. 2009](#), [Quevedo and Zuniga 2010](#), [Kim, Choi et al. 2013](#)).

The MOTS used four drugs (MSDK) combined in a unique formulation using specific dosages of each melatonin (5mg), strontium citrate (450mg), vitamin D<sub>3</sub> (2000IU) and vitamin K<sub>2</sub> (60mcg) and timed for intake at night to achieve maximal therapeutic efficacy and to minimize adverse effects. The 60mcg dose of vitamin K<sub>2</sub> was expected to minimize any possible risk of blood coagulation although no such adverse effects are reported in clinical studies using MK7 at a higher dose and for a longer time ([Kanellakis, Moschonis et al. 2012](#), [Knapen, Drummen et al. 2013](#)). Unlike the COMB study, the MOTS also targeted the more fracture-prone stage of bone loss—osteopenia. Moreover, these compounds of MSDK share common signaling pathways involved in osteoblastogenesis, osteoclastogenesis and bone mineralization (**Figure 3**).

As shown in **Figure 3**, melatonin induces the expression of *RUNX2*, the master regulator of osteoblastogenesis and TGF- $\beta$ , BMP and/or Wnt/ $\beta$ -catenin ([Maria and Witt-Enderby 2014](#)). Melatonin, like strontium, can also govern the lineage allocation of mesenchymal stem cells from adipocytes to osteoblasts through the MAPKs—MEK1/2 and MEK5 and through the metabolic proteins—PPAR $\gamma$  and GLUT4 ([Maria, Samsonraj et al. 2017](#)). Strontium works through the calcium sensing receptor in osteoblasts via activation of MAPK-ERK1/2 and Wnt signaling, leading to increased bone formation, decreased adipogenesis and increased bone mass *in vivo* ([Saidak and Marie 2012](#)). Vitamin K<sub>2</sub> produces osteoblast-inducing effects via TGF- $\beta$  and the Wnt/ $\beta$ -catenin pathway ([Yamaguchi 2014](#)). Both melatonin and strontium decrease RANKL in osteoblast precursors resulting in an inhibition of osteoclast formation ([Koyama, Nakade et al. 2002](#), [Saidak and Marie 2012](#)). For melatonin, these inhibitory effects on osteoclast differentiation are dependent upon contact with osteoclasts ([Maria, Samsonraj et al. 2017](#)). Vitamin K<sub>2</sub> also

antagonizes RANKL-induced activation of the NFκB pathway, which is crucial for osteoclastogenesis (Yamaguchi 2014). An exception to these compounds is vitamin D<sub>3</sub>, which induces both osteoblast and osteoclast to balance bone remodeling and promote mineralization (Anderson, Lam et al. 2013, Nahas-Neto, Cangussu et al. 2017).



**Figure 3. Regulation of signaling pathways in osteoblast and osteoclast and bone remodeling by melatonin (Mel), strontium (Sr), vitamins D<sub>3</sub> and K<sub>2</sub>.** Stimulation of MAPKs (ERK1/2, p38 or JNK) pathways by melatonin and strontium, TGF-β by melatonin and vitamin K<sub>2</sub> and Wnt/β-catenin pathways by melatonin, strontium and vitamin K<sub>2</sub>— all lead to increased expression of osteogenic genes (*RUNX2*, *BMP2*, *OSTERIX* and *OSTEOCALCIN*) and subsequent osteoblast differentiation and bone formation. RANKL, released from osteoblast progenitors, binds to RANK on osteoclast precursors and recruits the adaptor protein, TRAF6. TRAF6 then activates multiple

signaling cascades including: NF $\kappa$ B, Akt/PKB, mTOR and MAPKs (JNK, ERK, and p38) resulting in osteoclast differentiation and bone resorption. Inhibition of RANKL by melatonin or strontium inhibits osteoclastogenesis and bone resorption. Vitamin K<sub>2</sub> specifically blocks the NF $\kappa$ B pathway in osteoclasts and inhibits resorption. Vitamin D<sub>3</sub> primarily enhances mineralization by increasing calcium uptake. It also supports both bone formation and resorption in an optimal way, aiding in balanced bone remodeling. Furthermore, strontium and vitamin K<sub>2</sub> improve bone microarchitecture, aiding in bone quality. TGF= transforming growth factor, MEK1/2= MAP kinase/ERK kinase, ERK1/2= extracellular signal-regulated kinase, JNK= c-Jun N-terminal kinases, ALP= alkaline phosphatase, RUNX2= runt-related transcription factor 2, BMP-2= bone morphogenetic protein 2, Wnt= wntless type, M-CSF= macrophage colony-stimulating factor, c-Fms= colony-stimulating factor 1 receptor, RANKL= receptor activator of NF $\kappa$ B ligand, RANK= receptor activator of NF $\kappa$ B, OPG= osteoprotegerin, TRAF6= TNF receptor-associated factor, mTOR= mammalian target of rapamycin, Akt/ PKB= protein kinase B, NFATc1= nuclear factor of activated t cells calcineurin-dependent 1, TRAP= tartrate-resistant acid phosphatase, CTR= calcitonin receptor, GSK= glycogen synthase kinase, R-Smad= receptor-regulated Smad, Co-Smad= common mediator Smad, OCN= osteocalcin. Figure adapted from Maria *et al* 2014 ([Maria and Witt-Enderby 2014](#)).

In addition to improving bone health outcomes in people, a recent budget impact analysis determines that the introduction of melatonin into an osteoporosis treatment formulary may result in a saving of \$1.3 million in annual treatment costs ([Bondi, Khairnar et al. 2015](#)), thereby lowering the economic burden associated with bone loss therapies. All these findings provide the rationale for the testing of MSDK as a potential therapeutic candidate for postmenopausal osteopenia.



## 1.8. Hypothesis

Combination therapy using melatonin, strontium citrate, vitamin D<sub>3</sub> and vitamin K<sub>2</sub> will improve bone formation and prevent bone loss by increasing osteoblast activity, by reducing osteoclast activity and by improving the overall health-related QOL in postmenopausal women with osteopenia.

## 1.9. Specific aims

A translational research approach was taken using both clinical and *in vitro* hMSCs/hPBMCs co-culture models to test the study hypothesis. The clinical component of this translational research study involved the assessment of MSDK's effect on bone health and QOL in postmenopausal osteopenic women whereas the *in vitro* component of this study assessed the molecular mechanisms underlying the clinical effects of MSDK therapy, particularly focusing on its role in regulating the activity of osteoblasts and osteoclasts. The specific aims include:

1. Assess the effects of melatonin/strontium citrate/vitamin D<sub>3</sub>/vitamin K<sub>2</sub> (MSDK) on bone density, bone marker turnover and fracture risk in postmenopausal osteopenia.
2. Assess the effects of melatonin/strontium citrate/ vitamin D<sub>3</sub>/vitamin K<sub>2</sub> (MSDK) on menopause-specific quality of life, perceived stress, anxiety, depression and general well-being in postmenopausal osteopenia.
3. Assess the effects of melatonin/strontium citrate/vitamin D<sub>3</sub>/vitamin K<sub>2</sub> (MSDK) on human bone cells (osteoblast and osteoclast) viability and activity *in vitro* in hMSCs/hPBMCs co-cultures grown as transwell or layered.

## **Chapter 2: MOTS clinical trial— Assessing the effects of melatonin, strontium citrate, vitamin D<sub>3</sub> and vitamin K<sub>2</sub> (MSDK) on bone health (bone density, bone markers turnover, fracture risk and health-related quality of life in postmenopausal osteopenia**

### **2.1. Materials and Methods**

#### **2.1.1. Recruitment and enrollment**

The clinical component of this translational study was designed as a randomized double-blind placebo-controlled one-year trial and referred to as “Melatonin-micronutrient Osteopenia Treatment Study (MOTS)”. All study-related investigations were conducted in accordance with the ethical standards and following the Declaration of Helsinki and national and international guidelines. The clinical trial received IRB approval by the Duquesne University Institutional Review Board on May 23, 2013 (IRB Grant protocol number 13-59) and was documented at the [clinicaltrials.gov](http://clinicaltrials.gov) on June 5, 2013 (Identification no.: NCT01870115) (see **Appendix I**).

Study subject recruitment was initiated soon after the study received IRB approval and the MOTS was approved by [clinicaltrials.gov](http://clinicaltrials.gov). Strategies for recruiting participants included publishing of study-related articles in neighborhood and city newspapers (e.g. South Hills Almanac and Pittsburgh Post-Gazette) and in the Duquesne University’s DU Daily website, advertising on Craigslist and posting of flyers around the Pittsburgh community. Samples of materials used for recruitment are shown in **Appendix II**. During the phone interview, potential participants were first informed in detail about the study expectations and procedures and then were screened for their eligibility criteria.

Inclusion criteria included being postmenopausal with osteopenia (T-score between -1 to -2.5), willingness to participate in a 12-month study, willingness to take the therapy right before

bed, must not consume alcohol with the medication, willingness to undergo testing of bone markers and other biochemical parameters before and after the drug therapies, and willingness to provide a self-assessment on the quality of life throughout the program. Once all the inclusion criteria were satisfied, subjects were screened further and eliminated based on our exclusion criteria. Exclusion criteria primarily focused on eliminating the factors that might have any positive or negative impact on bone health and quality of life or could interfere with the treatment effects. Women who already developed osteoporosis (T-score less than -2.5), while being on medication or not, were excluded because our study intent to examine the treatment effect on osteopenia. Also, if bone deterioration proceeds towards an advanced stage of bone loss leading to osteoporosis, established pharmacotherapy is required to avoid serious consequences such as fracture. Other exclusion criteria included: women who developed osteopenia because of other medical conditions such as hyperparathyroidism, metastatic bone disease, multiple myeloma or chronic steroid use; and women who were recently taking any medications or treatment that could potentially improve or deteriorate bone health and affect their quality of life. These included: hormone therapy (HT), birth control pills, prescription medications for bone loss such as bisphosphonates, sleep, depression or anxiety, ulcerative colitis or regulation of blood pressure; and steroids used either recently or chronically for the past 6 months. Moreover, women with other medical conditions such as uncontrolled hypertension, moderate or severe hepatic impairment, severe sleep apnea, chronic obstructive pulmonary disease, and current use of tobacco were excluded from the study. The rationale for choosing these criteria has been discussed further in the Discussion.

Individuals who satisfied both the inclusion and exclusion criteria were invited to schedule an initial visit with the study team at the Center for Pharmacy Care at Duquesne University. Prior

to the visit, an information package was sent to their addresses. This package contained the consent form in which all necessary study-related information were described (e.g., purpose of the study, risks and benefits of joining the study, possible side effects of the medications, study confidentiality and participant's rights) along with the study team contact information. The package also contained a welcome letter, directions and map to the Center for Pharmacy Care at Duquesne University and a sheet containing frequently asked questions. The information packet was sent beforehand to provide sufficient time for the potential participants to review the study documents and to familiarize themselves with the overall study plan.

At the first visit, participants' osteopenia was confirmed by their recent DXA report. Free DXA scans were given to the potential participants who fulfilled all other eligibility criteria but did not present a recent DXA report. Osteopenia was also verified by heel ultrasonography using the Achilles Insight Ultrasonometer (GE Healthcare, USA) even though the heel ultrasonography was only used to assess its validity against DXA. Blood pressure readings were taken from both arms to ensure that participants had an average blood pressure between 140/90 and 100/60. Women who fulfilled the bone density and blood pressure requirements were invited to enroll in the study. Women who were excluded because of the DXA scores revealing osteoporosis (T-score <-2.5), high blood pressure or any other serious medical issues were encouraged to contact their primary care physician. Enrolled participants then completed a baseline intake form (**Figure 4**) detailing basic demographic information and consumption of any prescription and nonprescription drugs and/or supplements. An experienced registered nurse blinded to the group assignments performed the study-related health assessments and blood draws. Participants were randomly assigned to the MSDK or placebo groups based on a block randomization design (see section **2.1.2.**) and were then given an adequate supply of study capsules and diary pages for two months. Participants also

were given necessary instructions about pill intake, diary pages and study-related appointments. An initial visit form was prepared to crosscheck if all the procedures were performed correctly (see **Appendix III**). After the interview, the form was completed by the study coordinator and signed by the principal investigator.

BASELINE INTAKE FORM

ID \_\_\_\_\_

**A. Personal Information**

Date:

First name:  Last name:  M.I.:

Address:

City:  State:  Zip:

Phone:  or  |

Email:

Best time to contact:

Date of Birth (MM/DD/YYYY):  /  /  Age

How did you find out about this study?  Flyer  DU Daily  Advertisement  
 Friend  Other \_\_\_\_\_

**B. Background Information**

Are you a postmenopausal woman?  Yes  No  
• If yes, when did you first realize? \_\_\_\_\_

Have you had at least one period in the last one year?  Yes  No  
• If yes, date of last period \_\_\_\_\_

Are you currently using any hormone replacement therapy or hormone birth control such as oral medications, creams, gels, patches, vaginal suppositories or injectables?  Yes  No

Have you been diagnosed with osteopenia?  Yes  No  
\* What is osteopenia? Osteopenia refers to having bone mineral density (BMD) that is below normal levels, but not low enough to be classified as osteoporosis.  
• If yes,  
When? \_\_\_\_\_  
Did you have bone mineral test? Which ones? \_\_\_\_\_

*Figure 4. Baseline intake form*

BASELINE INTAKE FORM

ID \_\_\_\_\_

|   |  |
|---|--|
| Have you been diagnosed with osteoporosis?<br><i>* What is osteoporosis? Osteoporosis refers to a greater loss of bone mineral density (BMD) compared to osteopenia.</i><br>• If yes, When? _____<br>What diagnostic test was used? _____                 | <input type="checkbox"/> Yes <input type="checkbox"/> No   |
| Are you currently taking prescription medications for thinning bones such as Bisphosphonates, Calcitonin etc.?<br><i>Examples: Fosamax, Actonel, Boniva, Aredia, Reclast</i><br>• Have you taken any of these types of medications in the last 12 months? | <input type="checkbox"/> Yes <input type="checkbox"/> No<br><br><input type="checkbox"/> Yes <input type="checkbox"/> No |
| Are you currently taking any prescription sleep aids?<br><i>Examples: ramelteon (Rozerem), eszopiclone (Lunesta), zolpidem (Ambien), zaleplon (Sonata), triazolam (Halcion)</i>   | <input type="checkbox"/> Yes <input type="checkbox"/> No   |
| Are you currently taking any prescription medication for depression, stress or anxiety?<br><i>Examples: fluvoxamine (Luvox), sertraline (Zoloft), escitalopram (Lexapro)</i>  | <input type="checkbox"/> Yes <input type="checkbox"/> No   |
| Are you currently taking any medication to control blood pressure?<br><i>Examples: atenolol (Tenormin), metoprolol (Lopressor), propranolol (Inderal)</i>   | <input type="checkbox"/> Yes <input type="checkbox"/> No   |
| Are you currently taking any medication for gastritis, acid reflux or ulcer?<br><i>Examples: H<sub>2</sub> blocker (acid, pepcid, tagamet, zantac), Proton pump inhibitor (prilosec, nexium, protonix, prevacid)</i>                                      | <input type="checkbox"/> Yes <input type="checkbox"/> No   |
| Do you have any history of heart disease, atherosclerosis (hardening of the artery) or stroke? Do you use Pacemaker? Do you have any strong family history of heart disease?  | <input type="checkbox"/> Yes <input type="checkbox"/> No   |
| Have you been diagnosed with Hyperparathyroidism, Multiple myeloma, Chronic obstructive pulmonary (lung) disease (COPD), Severe sleep apnea, Epilepsy or Cancer,  | <input type="checkbox"/> Yes <input type="checkbox"/> No   |
| Have you been diagnosed with deep venous thrombosis or reported leg blood clot?   | <input type="checkbox"/> Yes <input type="checkbox"/> No   |
| Do you have any medical condition associated with your liver?   | <input type="checkbox"/> Yes <input type="checkbox"/> No   |
| Are you a smoker?   | <input type="checkbox"/> Yes <input type="checkbox"/> No   |
| Are you allergic or sensitive to any foods, herbals or nutritional supplements?<br>• If yes, which ones? _____  | <input type="checkbox"/> Yes <input type="checkbox"/> No   |
| Do you have multiple chemical sensitivity (MCS)?<br>• If yes, which ones? _____   | <input type="checkbox"/> Yes <input type="checkbox"/> No   |

*Figure 4 (continued). Baseline intake form*

**C. Medications**

Are you currently taking any prescription (Rx) medications?  Yes  No

If yes, please list -

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

Are you currently taking any non-prescription or over-the-counter (OTC) medications?  Yes  No

If yes, please list -

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

**D. Supplements:**

Are you currently taking any nutritional supplements, vitamins, minerals or herbals?  Yes  No

If yes, please list -

\_\_\_\_\_

\_\_\_\_\_

\_\_\_\_\_

*Figure 4 (continued). Baseline intake form*

**2.1.2. Randomization and treatment allocation**

As determined by mixed model analysis and based on the means and standard deviations obtained for the serum bone marker data from the MOPS ([Kotlarczyk, Lassila et al. 2012](#)), a difference of 10% in T-scores (12 month-baseline; average of % increases in lumbar spine density) would be necessary to achieve a statistically significant result. Thus, a sample size of 10 per group would provide enough participants to detect a significant (i.e., 10%) change in lumbar bone density

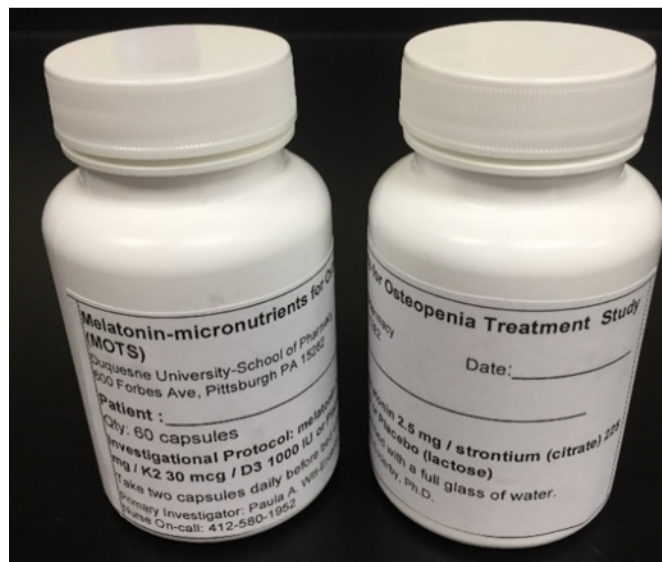


with 80% power, and an alpha of .05. Recruiting 12 per group factors in a 20% attrition rate. A computer-generated block randomization scheme was designed to assign the recruited participants (n=22) in either placebo (n=11) or MSDK (n=11) group. MSDK referred to the group receiving the medication. The block randomization method was chosen to preserve the balance in sample size between groups throughout the study period ([Suresh 2011](#)). In this process, participants were recruited in small blocks in a way that randomly allocated half of the participants to placebo and the other half to MSDK within each block. Random allocation was performed using a list of numbers generated with the statistical software Microsoft Excel (version 2012). Since there were only two treatment groups in our study (i.e. placebo and MSDK), a block size of 4 was utilized. Both the study subjects and investigators were blinded to the group assignments ensuring a double-blind structure and, upon enrollment, each participant received an identification number to maintain anonymity. Study participants were unblinded only after the treatment interventions had been completed and study data were analyzed. Since the MOTS followed a highly selective inclusion and exclusion criteria to avoid any bias, it took nearly 21 months to achieve the optimum number of participants. Allocation concealment was maintained among the participants and the MOTS team throughout the entire study ensuring the validity of study outcome.

### **2.1.3. Treatment regimen**

Treatment capsules, referred to as “MSDK” were formulated using 5 mg melatonin, 450 mg strontium citrate, 2000 IU vitamin D<sub>3</sub> and 60µg vitamin K<sub>2</sub> divided into two capsules. Therefore, each capsule contained melatonin 2.5 mg / strontium citrate 225 mg / K<sub>2</sub> (MK7) 30 mcg / D<sub>3</sub> 1000 IU). This dosage regimen was previously used by the naturopathy specialist Dr. Mark Swanson, where it demonstrated therapeutic efficacy. Besides, this study intended to use the compounds at a low therapeutic dose in the combination therapy MSDK to ensure safety while

maintaining efficacy. Identical placebo capsules matching in size, shape and color contained plant fiber. Study capsules were formulated and manufactured according to the principal investigators' specifications and supplied by Pure Encapsulations, Inc. (Sudbury, MA, USA). Both MSDK and placebo were supplied in identical pill bottles as shown in **Figure 5**. The groups were coded as A and B and only the graduate student on the study team was unblinded to this coding procedure and was responsible for delivering the pill bottles. Subjects were instructed to take two capsules by mouth daily at night at their usual bedtime. Pill bottles contained a medication label (see **Appendix IV**), where the dosing regimen, date of delivery and other necessary information about the study were clearly described. In addition to the study pills, participants were allowed to take <1000 IU of vitamin D<sub>3</sub> and 600 mg of calcium daily for ethical reasons; however, the amount and duration of their use were recorded by each participant through the daily diaries.



*Figure 5. Study medications (MSDK and Placebo) in identical bottles*

#### **2.1.4. Treatment follow-up**

After the first visit, bimonthly visits (at months 2, 4, 6, 8, 10 and 12) were arranged at the Center for Pharmacy Care at Duquesne University over a 12-month period. Study-related physical examinations were performed by the same nurse co-investigator to avoid procedural variation. Participants received a 2-month supply of medication and daily diaries at each visit to keep track of their daily pill intake, sleep duration, physical activity and any other information about their general well-being that the participant would feel was important to note. At visits 2-6, study participants were asked to bring with them their pill bottles from the preceding two months to be counted and the diary pages. Similar to initial visit form, month-specific visit forms were prepared to keep track of all completed assessments and signed by the principal investigator. An appointment card was given to each participant to carry with them during study visits so that it could be updated with the next appointment date, even though the graduate student called each participant to remind them about the visit at least a week prior to the appointment day. Usually the bimonthly visits were short, around 15-20 minutes, except the ones held at months 6 and 12. These two visits were long because blood samples were taken from the participants and participants were asked to complete the psychological questionnaires. Treatment follow-up ensured that there was no missing data for any participants. The study timeline is shown in **Table 1**.

#### **2.1.5. Participants' right and confidentiality of the study**

Emphasis was placed on preserving the subject's rights. For example, subjects could withdraw from the study at any time without any obligation. Attrition from the MOTS was minimized by having the study coordinator call each subject monthly to answer any questions or concerns. Participants were also allowed to contact the study coordinator at any time. A clinician in naturopathy, who was one of the co-investigators of this study, also reviewed the health record

of each participant. Alongside the free health assessments and two DXA scans, participants were also given a necessary compensation of \$80 for their time to answer the questionnaires, for the discomfort they experienced during the blood draws as well as to cover the costs for their transportation.

|   | Baseline | Month 2 | Month 4 | Month 6 | Month 8 | Month 10 | Month 12 |
|---|----------|---------|---------|---------|---------|----------|----------|
| <b>Bone health</b>  |          |         |         |         |         |          |          |
| Bone mineral density (BMD)                                      |          |         |         |         |         |          |          |
| ▪ Dual X-ray Absorptiometry (DXA)                               | ✓        |         |         |         |         |          | ✓        |
| ▪ Achilles ultrasonography                                      | ✓        | ✓       | ✓       | ✓       | ✓       | ✓        | ✓        |
| Fracture Risk (FRAX)  | ✓        |         |         |         |         |          | ✓        |
| <b>Bone Markers</b>   |          |         |         |         |         |          |          |
| ▪ Procollagen type 1 amino-terminal propeptide (PINP)           | ✓        |         |         | ✓       |         |          | ✓        |
| ▪ Osteocalcin (OC)  | ✓        |         |         | ✓       |         |          | ✓        |
| ▪ Collagen type I c-telopeptide (CTx)                           | ✓        |         |         | ✓       |         |          | ✓        |
| <b>Biochemicals</b>   |          |         |         |         |         |          |          |
| ▪ Melatonin   |          |         |         |         |         |          | ✓        |
| ▪ Vitamin D <sub>3</sub>  | ✓        |         |         |         |         |          | ✓        |
| ▪ C-reactive protein (CRP)                                      | ✓        |         |         |         |         |          | ✓        |
| <b>Quality of Life</b>  |          |         |         |         |         |          |          |
| Menopause Specific Quality of Life, Anxiety, Stress, Depression |          |         |         |         |         |          |          |
| ▪ Questionnaires (MENQOL, STAI, PSS, CES-D)                     | ✓        |         |         | ✓       |         |          | ✓        |
| General well-being and compliance                               |          |         |         |         |         |          |          |
| ▪ Daily diary (Sleep, mood, GI upset, aches/pains)              | ✓        | ✓       | ✓       | ✓       | ✓       | ✓        | ✓        |
| ▪ Pill count  | ✓        | ✓       | ✓       | ✓       | ✓       | ✓        | ✓        |

*Table 1: Experimental timeline of MOTS clinical trial*

Participant confidentiality was highly preserved throughout the study and while publishing the study data. A HIPAA compliant coding procedure was applied that provided for identification coding on all data collection forms. Information gathered from the participants were only accessible by the research team and were used for research purposes only. All written materials

and consent forms were stored in a locked file cabinet in the principal investigator's laboratory. Only the participants' responses but not their identity appeared in the statistical analysis as well as in the published paper ([Maria, Swanson et al. 2017](#)). Following IRB instructions, all research materials will be destroyed after 7 years following completion of the study.

#### **2.1.6. Bone density measurement**

Bone mineral density (BMD) was measured at baseline (month 0) and at month 12 in the left femoral neck, total left hip and lumbar spine L1-L4 area via Dual-energy X-ray absorptiometry (DXA). DXA is the most widely accepted and pain-free method for bone densitometry scanning and gives the most accurate T-score reading ([Imaging 2017](#)). Participants were given necessary instructions regarding where to go for their bone density assessments. DXA scans were performed using Hologic bone densitometer (Weinstein imaging, PA, USA). Changes in BMD ( $\text{g}/\text{cm}^2$ ) from baseline to month 12 were evaluated, compared between groups and reported as means  $\pm$  S.E.M.

Participant's calcaneus (heel) bone density T-scores were measured bimonthly using the Achilles InSight Ultrasonometer (GE Healthcare, USA) to assess how this measurement correlated with DXA. Achilles ultrasonography is a quick, comfortable and pain-free process in which the participants were asked to place their non-dominant foot in between the two membranes of the ultrasonometer. The membranes filled with warm water surrounding the foot and were then subjected to ultrasound through the heel. Just prior to testing, the heel area was thoroughly wet with ethanol spray to ensure proper signal transduction ([Healthcare](#)). The machine automatically created a result sheet containing the T-scores for the ease of interpretation. Mean ( $\pm$  S.E.M.) changes in heel T-score were calculated for each time point and compared within and between groups.

### 2.1.7. Fracture risk assessment

Fracture risk was assessed at months 0 and 12 using the FRAX<sup>®</sup>—a computer-generated algorithm (<http://www.shef.ac.uk/FRAX/>). The FRAX<sup>®</sup> estimates the 10year probability of a major osteoporotic fracture (clinical spine, forearm, hip or shoulder fracture) and a hip fracture in men and women based on their current femoral neck bone density, body composition, previous fractures, parental history of hip fracture and current risk factors ([Kanis, McCloskey et al. 2010](#), [Kanis, Hans et al. 2011](#)). A fracture assessment questionnaire (**Figure 6**) was prepared for collecting information required to calculate FRAX<sup>®</sup> scores from participants and the scores were calculated using the validated FRAX<sup>®</sup>- fracture risk assessment tool (**Figure 7**). Mean ( $\pm$  S.E.M.) changes in fracture risk from month 0 to month 12 were calculated and compared between groups.

FRACTURE RISK ASSESSMENT

ID \_\_\_\_\_

NAME (First, Last): \_\_\_\_\_ Date: \_\_\_\_\_

Age \_\_\_\_\_ Height \_\_\_\_\_ ft \_\_\_\_\_ in Weight \_\_\_\_\_ lbs

What is your race/ethnicity?

Caucasian  African-American  Hispanic  Asian  Other \_\_\_\_\_

Have you suffered from a fracture in your adult life?  Yes  No

- If yes, how did it occur? \_\_\_\_\_

\_\_\_\_\_

Has your mother or father ever had a hip fracture?

- Mother  Yes  No  Unknown
- Father  Yes  No  Unknown

Are you currently using any medications such as prednisone, cortisone, medrol dose pack, prednisolone, or dexamethasone?  Yes  No

- If yes, what is the dose? \_\_\_\_\_

Have you taken medications such as prednisone, cortisone, medrol dose pack, prednisolone, or dexamethasone in the last 3 months?  Yes  No

- If yes, what is the dose? \_\_\_\_\_

Have you been diagnosed with rheumatoid arthritis?  Yes  No

Do you currently smoke?  Yes  No

On average, do you consume more than 3 alcoholic drinks per day?  Yes  No

Do you have any of the following?

- Type I diabetes (insulin dependent)
- Osteogenesis imperfecta
- Untreated, long-standing hyperthyroidism
- Malabsorption
- Chronic liver disease

*Figure 6. Fracture risk (FRAX®) assessment questionnaire*

*Figure 7. Fracture risk (FRAX®) assessment tool*

### 2.1.8. Collection and storage of serum and urine samples

To assess bone turnover marker status (described in section 2.1.9. Biochemical assessments and other hormones associated with bone health, participants’ serum samples were collected at months 0, 6 and 12. Approximately 2mL of blood sample was taken via venous puncture using BD Vacutainer® Safety-Lok blood collection set with a 23-gauge needle (BD, USA) and collected in 8.5 mL BD Vacutainer® SST™ Plus blood collection tubes (BD, USA). Blood collection was performed by the registered nurse in the MOTS. Collected samples were allowed to clot for at least 30 min at room temperature. Serum was isolated by centrifugation (1200g for 25 min) and stored at -20°C until the time of analysis. To maintain the integrity of the serum samples, small aliquots



of each sample were frozen and stored. All samples were tested at the same time to minimize analytical variation.

For the melatonin measurement, participants' first morning urine were collected at the end of the study (month 12). Participants were asked to collect all their urine samples between 10pm and 6am the night preceding the day of their last visit and to freeze them. At their previous (month 10) visit, they were supplied two urine cups and ice packs to collect and store their urine and an instruction sheet detailing how to collect, store and transport the sample to the Center for Pharmacy Care.

### **2.1.9. Biochemical assessments**

***Bone markers.*** Bone marker turnover was assessed in serum sample at months 0, 6 and 12. Serum bone formation markers, total procollagen type 1 amino-terminal propeptide (P1NP) and osteocalcin (OC; both intact and N-terminal mid-fragments) were measured via sandwich enzyme linked immunosorbent assay (ELISA) assay using the human total P1NP ELISA kit (CAT# MBS2504819, Mybiosource, CA, USA) and osteocalcin (1-43/49) enzyme immunoassay assay kit (CAT# 43-OSNHU-E01, ALPCO Diagnostic, NH, USA), respectively, according to the manufacturer's instructions. The serum bone resorption marker, collagen type I c-telopeptide (CTx) was measured via sandwich ELISA assay using Human Cross-linked Carboxy-terminal Telopeptide Of Type I Collagen (CTX-I) ELISA Kit (CAT# MBS700254, Mybiosource, CA, USA) following kit instructions. In the sandwich ELISA assays, micro ELISA plates pre-coated with an antibody specific to the desired bone marker (e.g. P1NP or OC or CTx) were used. When serum samples were added to the plate, the bone marker present in the sample bound with that specific antibody. Next, a biotinylated detection antibody, specific for the protein and Avidin-Horseradish Peroxidase (HRP) enzyme conjugate, was added to the plate and incubated following

the removal of unbounded components from the plate via washing. The bone marker was “sandwiched” between the two antibodies. Then substrate solution was added to initiate the enzyme-substrate reaction. The absorbance (OD) was proportional to the concentration of the desired bone marker present in the test sample. Absorbance readings of standards, controls and test samples were measured spectrophotometrically at a wavelength of 450 nm using the Perkin Elmer Victor 1420 Multilabel plate reader (Waltham, MA, USA). A standard curve was generated from the OD values for each assay using the four-parameter logistic curve fit function. Concentrations were then calculated from the generated standard curves using Workout 2.0 software (Waltham, MA, USA). Mean ( $\pm$  S.E.M.). Changes in the concentration of bone markers P1NP (in pg/mL), OC (in ng/mL) and CTx (in ng/mL) were calculated for each time point and compared within and between groups. Ratios of bone resorption to bone formation (i.e. CTx:P1NP and CTx:OC) were calculated over time and compared within and between groups. All controls contained within each of the kits were within normal ranges.

**Melatonin.** Participants’ nocturnal urinary melatonin levels were measured at month 12 to investigate the therapeutic range of melatonin contained within MSDK required to attain an effect on bone. Urinary melatonin levels (in ng/mL/hr) were calculated via sandwich ELISA using the Melatonin-Sulfate Urine ELISA kit (CAT# RE54031, IBL International, Germany) per kit instructions and then mean ( $\pm$  S.E.M.) concentrations were compared between groups.

**Vitamin D<sub>3</sub>.** Serum vitamin D<sub>3</sub> levels were measured at months 0 and 12 by ELISA using the 25(OH) Vitamin D ELISA kit (CAT# ADI-900-215-0001, Enzo Life Science, NY, USA), according to the manufacturer’s instructions. This kit offered a competitive ELISA assay which detected the 25(OH) Vitamin D metabolite as an indicator for total Vitamin D concentration. Mean

( $\pm$  S.E.M.) concentrations of vitamin D<sub>3</sub> (in ng/mL) were calculated for each time point and compared between groups.

***C-reactive protein (CRP)***. Serum CRP levels were measured at months 0 and 12 by sandwich ELISA using the High sensitivity human C-reactive protein (hsCRP) ELISA kit (CAT# EKD01009, Biomatik LLC., DE, USA), following kit instructions. Following the assessment of absorbance (OD) values, mean ( $\pm$  S.E.M.) concentrations of CRP (in ng/mL) were calculated for each time point and compared between groups.

#### **2.1.10. Blood pressure and body composition**

Participants' blood pressure was measured at the initial visit (month 0) as a screening tool for the inclusion criteria and then every two months until the end of the study to assess the effect of MSDK on blood pressure. Systolic and diastolic blood pressure measurements were taken by the MOTS nurse on three separate occasions from the right and left arms. Mean ( $\pm$  S.E.M.) blood pressure readings at each time point were calculated and compared within and between groups. To avoid experimental variation, the same blood pressure cuffs was used in all assessments.

To assess the effect of MSDK on body composition, morphometric analysis was carried out at baseline (month 0) and at the end of the study (month 12) using TANITA™ body composition analyzer (Model# TBF-215, IL, USA). Participants were asked to stand on the analyzer and the machine automatically measure the participants' height, weight, body mass index (BMI), basal metabolic rate (BMR), fat percentage, fat mass (FM), lean body mass (FFM) and total body water (TBW). Mean ( $\pm$  S.E.M.) changes in body composition from baseline to month 12 were calculated and compared within and between groups.

### 2.1.11. Psychometric analyses

The effect of MSDK on subjective measures were assessed using four validated questionnaires: Menopause Specific Quality of Life (MENQOL) ([Lewis, Hilditch et al. 2005](#), [Hilditch, Lewis et al. 2008](#)), Spielberger's State-Trait Anxiety Inventory (STAI) ([Spielberger, Gorsuch et al. 1970](#), [Yu and Ho 2010](#)), Cohen's Perceived Stress Scale (PSS) ([Cohen, Kamarck et al. 1983](#), [Cohen 1988](#), [Yu and Ho 2010](#)) and the Center for Epidemiologic Studies Depression (CES-D) ([Radloff 1977](#)). Questionnaires were administered to the study cohort at baseline and then at months 6 and 12. A quiet and isolated environment was provided to the participants so that they could fill out the questionnaires without any distraction. Participants were asked to complete all questions so that there were no missing or incomplete data. Samples of psychometric questionnaires are shown in Figures 8 and 9.

***Menopausal quality of life:*** Menopause Specific Quality of Life (MENQOL) questionnaire (**Figure 8**) is a widely used validated tool that measures self-perceived quality of life related to menopausal health in middle aged women ([Lewis, Hilditch et al. 2005](#), [Hilditch, Lewis et al. 2008](#)). The concept of MENQOL relies on the fact that menopause brings a sudden change in a woman's life by producing symptoms that may disrupt her physical, emotional and social well-being. MENQOL contains 32 menopausal symptoms- related items, which are sub-divided into four domains: vasomotor (items no. 1-3), psychosocial (items no. 4-10), physical (items no. 11-26, 30-32) and sexual (items no. 27-29). In the questioning process for MENQOL, participants first identify if they have experienced a specific symptom (i.e. item) in the previous month by answering "yes" or "no". If they answer "no", they then move to the next item. If they answer "yes", then they are further asked to rate the symptom on a scale of 0 to 6 based on how much they were bothered by that item (0 means "not bothered" and 6 means "extremely bothered").

MENQOL scoring was performed according to the established guidelines ([Lewis, Hilditch et al. 2005](#), [Hilditch, Lewis et al. 2008](#)). Briefly, the items were scored on a scale of 1 to 8—1 corresponded to answer “no”, 2 corresponded to answer “yes” but not bothered, and a score of 3 to 8 depending upon how bothered the women was by the item; a value to 8 corresponded to “yes” with maximum botheration. Each domain was scored separately, and a high score indicates that the item was bothersome to the participant. Mean ( $\pm$  S.E.M.) scores of MENQOL domains were calculated for each time point and compared within and between groups.

*Anxiety*: Spielberger’s State-Trait Anxiety Inventory (STAI) is a widely used validated 40 item questionnaire that assess the intensity of anxiety-related feelings ([Spielberger, Gorsuch et al. 1970](#)), and, more recently, may be helpful in detecting certain mental disorders such as depression ([Kvaal, Ulstein et al. 2005](#)). The STAI questionnaire is sub-divided into two forms: form Y-1 measures state anxiety which refers to the short-term or transient level of anxiety (items no. 1-20) and form Y-2 measures trait anxiety referring to the long-term or enduring level of anxiety (items no. 21-40) ([Spielberger, Gorsuch et al. 1970](#)). Half of the items in each form indicate the presence of anxiety symptoms and the other half indicates the absence of anxiety symptoms. Participants’ scores were rated on a scale of 1 to 4 depending on the intensity of anxiety; a high score indicates more anxiety. For state form (Y-1), which measured how the participant was feeling at that moment or felt at the recent past, a score of 1 corresponded to “not at all” whereas 4 corresponded to “very much so”. For trait items (Y-2), which measured how the participant generally felt, the response scale went from 1 to 4 (1= “almost never, 4= “almost always”). Items that indicated an absence of anxiety were scored inversely. A total score ranging from 20-80 was obtained by summing up the responses on all items ([Spielberger, Gorsuch et al. 1970](#), [Yu and Ho 2010](#)). Mean ( $\pm$  S.E.M.) scores of STAI were compared over time within and between groups.

**Stress:** The Perceived Stress Scale (PSS) is another validated questionnaire (**Figure 9**) used to predict the degree to which one perceives stressful life events ([Cohen, Kamarck et al. 1983](#), [Cohen 1988](#)). The version utilized in this study had 10 questions measuring how often the participants encountered psychological stress during the last month (i.e. “how often they felt that they were unable to control any situation?”). There were some positive questions such as “how confidently they overcame any situation in the last month?”([Cohen 1988](#)). Participants were asked to rate the stressful events based on a 5-point scale (0= “never” to 4= “very often”); a high score indicates the presence of stress. Positive questions were scored inversely. Responses on all items were summed up to obtain a total score ranging from 0 to 40 as described. ([Cohen, Kamarck et al. 1983](#), [Yu and Ho 2010](#)). Mean ( $\pm$  S.E.M.) scores of PSS were compared over time within and between groups.

**Depression:** The Center of the Epidemiological Study of Depression Scale (CES-D) is a validated 20-item instrument that detects one’s current level of depressive symptoms including: depressed mood, loss of appetite, feeling of guiltiness and failure, lack of hope and ambitions, and forgetfulness in an adult community sample ([Radloff 1977](#)). Participants were asked to rate each of these symptoms based on a 4-point scale depending on their frequency of occurrence (0 = “rarely or none of the time or less than 1 day” and 3 = “most or all of the time: 5 to 7 days”). The severity of the depressive state was measured based on their total scores ranging from 0 to 60 as described, where the positive items were scored inversely ([Radloff 1977](#), [Yu and Ho 2010](#)). Mean ( $\pm$  S.E.M.) scores of CES-D were compared over time within and between groups.

### Menopause Specific Quality of Life (MENQOL) questionnaire

NAME (First, Last): \_\_\_\_\_ Date: \_\_\_\_\_ Age: \_\_\_\_\_

For each of the following items, indicate whether you have experienced the problem in the **PAST MONTH**. If you have, rate how much you have been bothered by the problem.

|  |                             | Not at all <span style="display: inline-block; width: 100px; border-bottom: 1px solid black;"></span> → Extremely |                          |                          |                          |                          |                          |                          |
|--|-----------------------------|---|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
|  |                             | bothered <span style="display: inline-block; width: 100px; border-bottom: 1px solid black;"></span> bothered      |                          |                          |                          |                          |                          |                          |
|  |                             | 0   | 1                        | 2                        | 3                        | 4                        | 5                        | 6                        |
| 1. HOT FLUSHES OR FLASHES                | <input type="checkbox"/> No | <input type="checkbox"/> Yes  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 2. NIGHT SWEATS                          | <input type="checkbox"/> No | <input type="checkbox"/> Yes  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 3. SWEATING                              | <input type="checkbox"/> No | <input type="checkbox"/> Yes  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 4. DISSATISFACTION WITH MY PERSONAL LIFE | <input type="checkbox"/> No | <input type="checkbox"/> Yes  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 5. FEELING ANXIOUS OR NERVOUS            | <input type="checkbox"/> No | <input type="checkbox"/> Yes  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 6. POOR MEMORY                           | <input type="checkbox"/> No | <input type="checkbox"/> Yes  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 7. ACCOMPLISHING LESS THAN I USED TO     | <input type="checkbox"/> No | <input type="checkbox"/> Yes  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 8. FEELING DEPRESSED, DOWN OR BLUE       | <input type="checkbox"/> No | <input type="checkbox"/> Yes  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 9. BEING IMPATIENT WITH OTHER PEOPLE     | <input type="checkbox"/> No | <input type="checkbox"/> Yes  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 10. FEELINGS OF WANTING TO BE ALONE      | <input type="checkbox"/> No | <input type="checkbox"/> Yes  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 11. FLATULENCE (WIND) OR GAS PAINS       | <input type="checkbox"/> No | <input type="checkbox"/> Yes  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 12. ACHING IN MUSCLES AND JOINTS         | <input type="checkbox"/> No | <input type="checkbox"/> Yes  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 13. FEELING TIRED OR WORN OUT            | <input type="checkbox"/> No | <input type="checkbox"/> Yes  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 14. DIFFICULTY SLEEPING                  | <input type="checkbox"/> No | <input type="checkbox"/> Yes  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 15. ACHES IN BACK OF NECK OR HEAD        | <input type="checkbox"/> No | <input type="checkbox"/> Yes  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
| 16. DECREASE IN PHYSICAL STRENGTH        | <input type="checkbox"/> No | <input type="checkbox"/> Yes  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |

Figure 8. Menopause Specific Quality of Life (MENQOL) questionnaire

MENQOL questionnaire *continued*

|   |                          | Not at all <span style="display: inline-block; width: 100px; border-bottom: 1px solid black;"></span> → Extremely<br>bothered <span style="float: right;">bothered</span> |                          |                          |                          |                          |                          |                          |
|---|--------------------------|---|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|
|   |                          | 0   | 1                        | 2                        | 3                        | 4                        | 5                        | 6                        |
| 17. DECREASE IN STAMINA                               | <input type="checkbox"/> | <input type="checkbox"/>  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|   | No                       | Yes   | 0                        | 1                        | 2                        | 3                        | 4                        | 5                        |
| 18. LACK OF ENERGY                                    | <input type="checkbox"/> | <input type="checkbox"/>  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|   | No                       | Yes   | 0                        | 1                        | 2                        | 3                        | 4                        | 5                        |
| 19. DRY SKIN  | <input type="checkbox"/> | <input type="checkbox"/>  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|   | No                       | Yes   | 0                        | 1                        | 2                        | 3                        | 4                        | 5                        |
| 20. WEIGHT GAIN                                       | <input type="checkbox"/> | <input type="checkbox"/>  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|   | No                       | Yes   | 0                        | 1                        | 2                        | 3                        | 4                        | 5                        |
| 21. INCREASED FACIAL HAIR                             | <input type="checkbox"/> | <input type="checkbox"/>  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|   | No                       | Yes   | 0                        | 1                        | 2                        | 3                        | 4                        | 5                        |
| 22. CHANGES IN APPEARANCE, TEXTURE OR TONE OF MY SKIN | <input type="checkbox"/> | <input type="checkbox"/>  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|   | No                       | Yes   | 0                        | 1                        | 2                        | 3                        | 4                        | 5                        |
| 23. FEELING BLOATED                                   | <input type="checkbox"/> | <input type="checkbox"/>  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|   | No                       | Yes   | 0                        | 1                        | 2                        | 3                        | 4                        | 5                        |
| 24. LOW BACKACHE                                      | <input type="checkbox"/> | <input type="checkbox"/>  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|   | No                       | Yes   | 0                        | 1                        | 2                        | 3                        | 4                        | 5                        |
| 25. FREQUENT URINATION                                | <input type="checkbox"/> | <input type="checkbox"/>  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|   | No                       | Yes   | 0                        | 1                        | 2                        | 3                        | 4                        | 5                        |
| 26. INVOLUNTARY URINATION WHEN LAUGHING OR COUGHING   | <input type="checkbox"/> | <input type="checkbox"/>  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|   | No                       | Yes   | 0                        | 1                        | 2                        | 3                        | 4                        | 5                        |
| 27. DECREASE IN MY SEXUAL DESIRE                      | <input type="checkbox"/> | <input type="checkbox"/>  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|   | No                       | Yes   | 0                        | 1                        | 2                        | 3                        | 4                        | 5                        |
| 28. VAGINAL DRYNESS                                   | <input type="checkbox"/> | <input type="checkbox"/>  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|   | No                       | Yes   | 0                        | 1                        | 2                        | 3                        | 4                        | 5                        |
| 29. AVOIDING INTIMACY                                 | <input type="checkbox"/> | <input type="checkbox"/>  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|   | No                       | Yes   | 0                        | 1                        | 2                        | 3                        | 4                        | 5                        |
| 30. BREAST PAIN OR TENDERNESS                         | <input type="checkbox"/> | <input type="checkbox"/>  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|   | No                       | Yes   | 0                        | 1                        | 2                        | 3                        | 4                        | 5                        |
| 31. VAGINAL BLEEDING OR SPOTTING                      | <input type="checkbox"/> | <input type="checkbox"/>  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|   | No                       | Yes   | 0                        | 1                        | 2                        | 3                        | 4                        | 5                        |
| 32. LEG PAINS OR CRAMPS                               | <input type="checkbox"/> | <input type="checkbox"/>  | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> | <input type="checkbox"/> |
|   | No                       | Yes   | 0                        | 1                        | 2                        | 3                        | 4                        | 5                        |

*Figure 8 (continued). Menopause Specific Quality of Life (MENQOL) questionnaire*

MENQOL reprinted from *Maturitas*, 50(3), Lewis JE, Hilditch JR, Wong CJ, Further psychometric property development of the Menopause-Specific Quality of Life questionnaire and development of a modified version, MENQOL-Intervention questionnaire, pp 209-221, copyright 2005, with permission from Elsevier.



## PERCEIVED STRESS SCALE

The questions in this scale ask you about your feelings and thoughts during the last month. In each case, you will be asked to indicate by circling *how often* you felt or thought a certain way.

Name \_\_\_\_\_ Date \_\_\_\_\_

Age \_\_\_\_\_ Gender (Circle): M F Other \_\_\_\_\_

0 = Never 1 = Almost Never 2 = Sometimes 3 = Fairly Often 4 = Very Often

1. In the last month, how often have you been upset because of something that happened unexpectedly? 0 1 2 3 4
2. In the last month, how often have you felt that you were unable to control the important things in your life? 0 1 2 3 4
3. In the last month, how often have you felt nervous and "stressed"? 0 1 2 3 4
4. In the last month, how often have you felt confident about your ability to handle your personal problems? 0 1 2 3 4
5. In the last month, how often have you felt that things were going your way? 0 1 2 3 4
6. In the last month, how often have you found that you could not cope with all the things that you had to do? 0 1 2 3 4
7. In the last month, how often have you been able to control irritations in your life? 0 1 2 3 4
8. In the last month, how often have you felt that you were on top of things? 0 1 2 3 4
9. In the last month, how often have you been angered because of things that were outside of your control? 0 1 2 3 4
10. In the last month, how often have you felt difficulties were piling up so high that you could not overcome them? 0 1 2 3 4

  
**mind garden**  
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www.mindgarden.com

#### References

The PSS Scale is reprinted with permission of the American Sociological Association, from Cohen, S., Kamarck, T., and Mermelstein, R. (1983). A global measure of perceived stress. *Journal of Health and Social Behavior*, 24, 386-396.  
Cohen, S. and Williamson, G. Perceived Stress in a Probability Sample of the United States. Spacapan, S. and Oskamp, S. (Eds.) *The Social Psychology of Health*. Newbury Park, CA: Sage, 1988.

*Figure 9. Perceived Stress Scale (PSS) questionnaire*

PSS is available for free download from:

<https://www.mindgarden.com/documents/PerceivedStressScale.pdf>

### 2.1.12. General well-being and treatment compliance as measured by daily diary

Participants' feeling of well-being and compliance to the treatment were assessed using a daily diary appropriate for the postmenopausal cohort (**Error! Reference source not found.**). The diary utilized in this study was a modified version of the daily diary used in our previous MOPS clinical trial ([Kotlarczyk, Lassila et al. 2012](#)). Participants were asked to record daily information regarding their sleep duration and experience, use of any prescription and non-prescription medications, exercise and general well-being. New diary pages were given to the participants at their bimonthly visits after collecting the old diary pages. Comments written on the diary pages were collated at the end of the study to see which themes emerged. In the MOTS, four categories emerged that included comments made about sleep quality, general mood, GI symptoms and general aches and pains (e.g. headache, ache in shoulder, ache in knees). Comments within each category were then sub-stratified as positive, negative or neutral. Comments related to an improvement in an existing physical condition or positive feelings were classified as positive; whereas comments that indicated worsening of any condition, the emergence of a new health problem, or negative (or sad) feelings were classified as negative. Comments that reflected indifferent thoughts such as those made about their daily activities or weather, or that did not reveal any health issue were classified as neutral. A study investigator blinded to the group assignments coded the comments as neutral= 0, negative= -1 and positive= +1. These comments were then summed up for each participant per group. Results for each category in each cohort (placebo and MSDK) were reported as percent of total comments per type per category per cohort. For data analyses, the positive and neutral comments made for each cohort (placebo vs. MSDK) were combined as per category and then compared to the negative comments made under the same category.

Sleep duration was determined by averaging the number of hours each participant slept at night. To assess if exercise had any confounding impact on participants' bone health, exercise intensity was determined according to the US Centers for Disease Control and Prevention (CDC) guidelines ([Medicine 2013](#)). Based on the type and intensity of exercise, participants fell into four different groups that were scored as: no exercise= 0; light exercise= 1; moderate exercise= 2; and high intensity or vigorous exercise= 3. According to the CDC, examples of moderate intensity exercise include brisk walking (3 miles per hour or faster, but not race-walking), water aerobics, bicycling slower than 10 miles per hour, tennis (doubles), ballroom dancing, general gardening etc. Examples of vigorous (high) intensity exercise include race walking (jogging or running), swimming laps, tennis (singles), aerobic dancing, bicycling 10 miles per hour or faster, jumping rope, heavy gardening (continuous digging or hoeing), hiking uphill or with a heavy backpack etc. ([Medicine 2013](#)). Sleep hours and exercise scores were compared between study cohort (placebo vs. MSDK) and reported as mean  $\pm$  S.E.M. Use of multivitamins/herbal/OTC supplements were also documented from their diary information and compared between groups. Other confounding factors were also assessed from the diary log that included: safety, tolerability and compliance to the treatment regimens. Compliance was also assessed at each visit through regular health assessments by the MOTS nurse and by counting the number of pills remaining from the last study visit.

Date: \_\_\_/\_\_\_/\_\_\_

Did you take your study medication today? Yes No  
If yes, what time? \_\_\_\_\_

Did you take any prescription medication today? Yes No  
If yes, please list \_\_\_\_\_  
\_\_\_\_\_

Did you take any non-prescription medication today such as:  
- Vitamin/mineral supplements? Yes No  
If yes, please list \_\_\_\_\_  
\_\_\_\_\_

- Herbal remedies? Yes No  
If yes, please list \_\_\_\_\_  
\_\_\_\_\_

- Other non-prescription medication? Yes No  
If yes, please list \_\_\_\_\_  
\_\_\_\_\_

Did you begin or end your period today? Yes No  
If yes, please circle begin or end

Approximately how many hours did you sleep last night? \_\_\_\_\_

Did you perform any physical activity/exercise today? Yes No  
If yes, please briefly describe activity including type of activity  
and approximate duration. \_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

Describe any additional comments you may have related to your general  
health or well-being today.  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_  
\_\_\_\_\_

**FOR OFFICE USE:**

ID \_\_\_\_\_ Month \_\_\_\_\_ Day \_\_\_\_\_

*Figure 10. Daily diary*

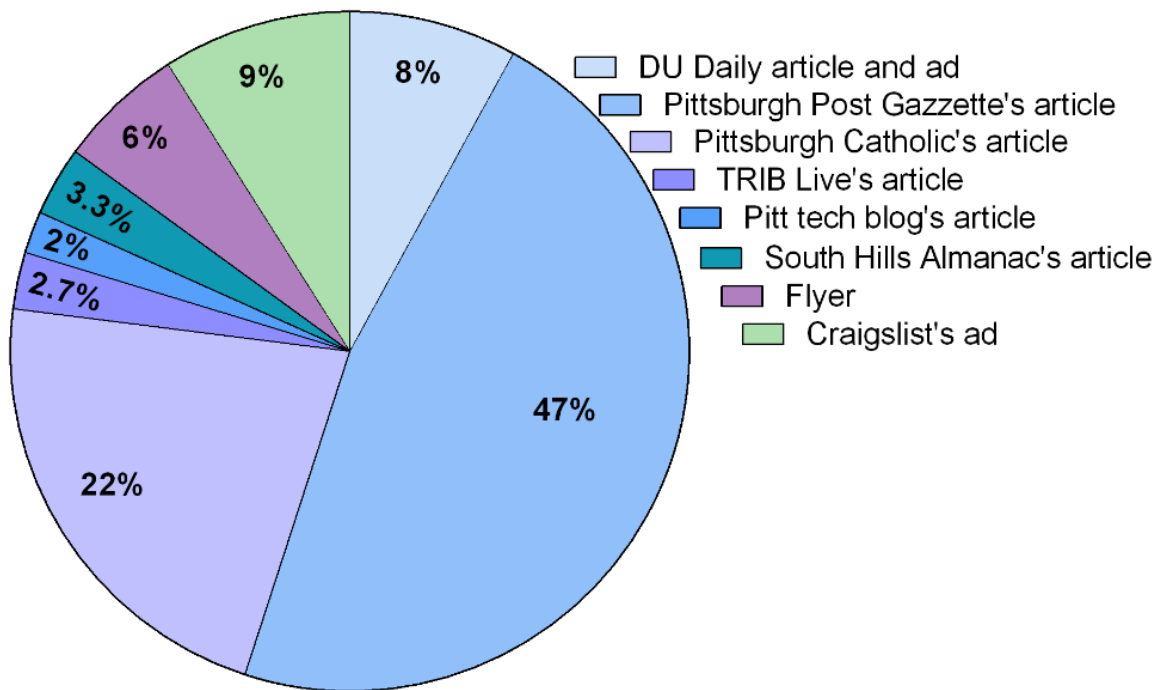
### 2.1.13. Statistical interpretations

To check if randomization was effective, a comparison of the baseline characteristics between MSDK and placebo groups were performed using Student's two-tailed *t*-test for independent samples with Welch's correction for unequal variances (continuous data) and Fishers exact test (categorical data). Mean changes and percentage mean changes from baseline to month 12 in continuous variables were compared between treatment groups using Student's one-tailed *t*-test for BMD measurements and Student's two-tailed *t*-test for all other endpoints with Welch's correction. Longitudinal analyses were carried out for the continuous variables with repeated measures (e.g. serum bone markers, vitamin D<sub>3</sub> and CRP levels, questionnaires, blood pressure and Achilles T-scores). Generalized linear mixed model (GLMM) approach was used to study the groups, the times, and the interaction between groups and times. In this analysis, groups and times were considered as fixed effects while subjects nested within the treatment groups were considered random. Comparisons of the groups over time were studied using orthogonal contrast. Pearson correlation was performed to analyze the correlation between age, melatonin, vitamin D<sub>3</sub> and CRP levels with the bone density, bone markers and morphometric changes. Dairy comments were analyzed using two-tailed Fishers exact test for two categorical outcomes. All statistical testing was carried out using JMP versions 12 (SAS Institute Inc., Cary, NC, USA) and GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA) for Macintosh. Primary and secondary endpoints were analyzed following the intention-to-treat (ITT) principle. Results were presented as mean ( $\pm$  S.E.M.) with significance considered at  $p < 0.05$ .

## 2.2. Results

### 2.2.1. Participant's recruitment and enrollment

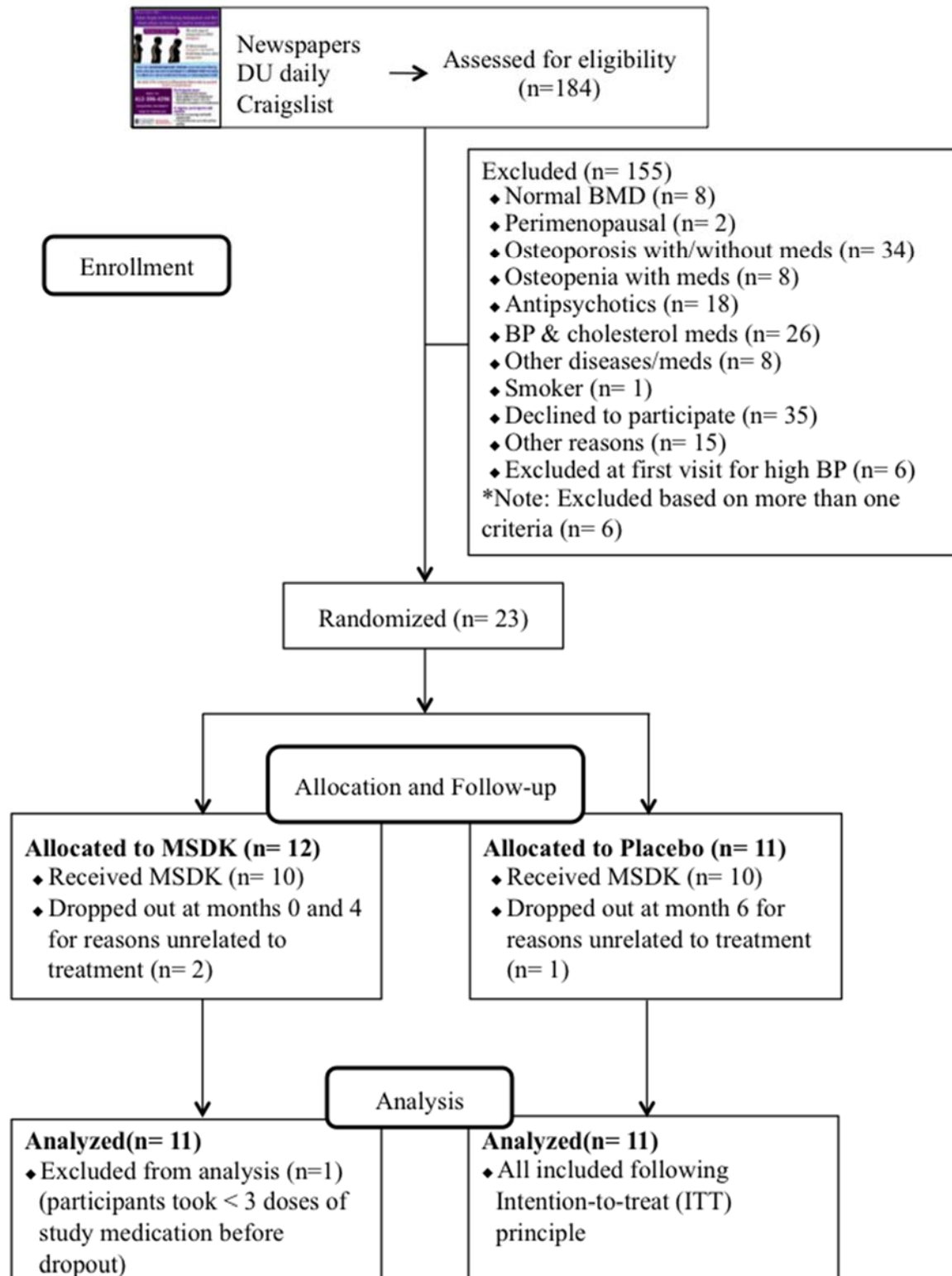
Nearly all recruitment strategies successfully generated interest among people from different areas of Pittsburgh as well as its nearest cities. As shown in **Figure 11. Responses to recruitment strategies employed in the study (n=184)**, an article published in the Pittsburgh Post-Gazette (PPG; see **Appendix IIB**) drew attention of nearly half of the women respondents.



**Figure 11. Responses to recruitment strategies employed in the study (n=184)**

The summary of participants' screening and enrollment process is illustrated in **Figure 12. Flow diagram of study subjects' recruitment and enrollment.** A total of 184 women responded to our advertisements, of which 105 (57.1%) did not meet the inclusion and exclusion criteria. Individuals were excluded due to having normal bone density T-score or not having a DXA scan before (7.6%); being perimenopausal (2%); being osteoporotic with or without taking medications (32.4%); or being osteopenic taking bisphosphonates or HT (7.6%). Other common reasons for

exclusion included taking medications that would influence our outcome measures. These included: use of blood pressure and cholesterol medications (25%) or medications for depression or anxiety (17%). If the respondent had diseases such as ulcerative colitis, rheumatoid arthritis (7.62%) or smoked (1%), then they were excluded as well. Among the 79 women invited to enroll in the MOTS, 29 (37%) accepted our invitation and attended their initial appointment to the study center, while another 50 individuals (63%) chose not to participate for their own reasons. These reasons included: fear of being on placebo for one year (35%) or not being able to commit bimonthly visits (25%). Fifteen women (31%) did not share their reasons for not participating in the study and another 6 osteopenic women (12.2%) did not want to stop taking their bone loss medications. At the initial visit and during the blood pressure screening, another 6 individuals were excluded for having hypertension, which they were completely unaware. At the end of the recruitment and screening process, 23 women were enrolled into the MOTS and randomized to receive either placebo (n=11) or MSDK (n=12) via block randomization, as described previously. One subject withdrew the day following enrollment and was not included in the overall analysis because it had been determined that if a subject took <3 dosages of the medication, then they would be replaced and not included in the analysis. Another two subjects withdrew (one from treatment and one from placebo) at month 4 and month 6. They were included in the analysis following intention-to-treat (ITT) principle. All women were self-identified as Caucasian.



*Figure 12. Flow diagram of study subjects' recruitment and enrollment.*



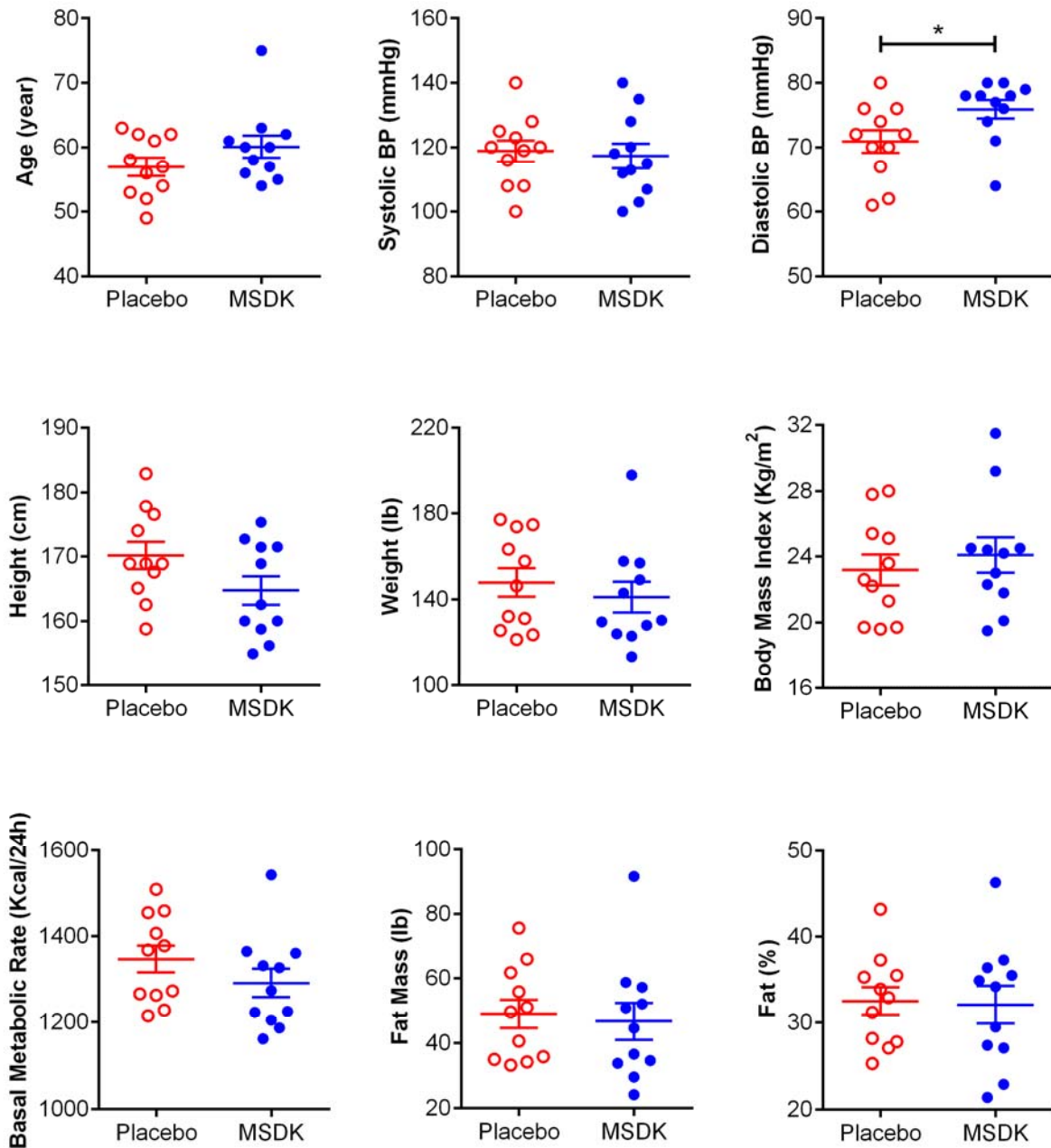
### **2.2.2. Similar baseline characteristics between groups ensured efficient randomization**

Our recruitment strategies resulted in a relatively healthy postmenopausal population (other than having osteopenia) with an age range [58.6 yr. (49 -75)]. Their blood pressure and body composition including height, weight, body mass index (BMI), basal metabolic rate (BMR), fat mass (FM), fat percentage (Fat%), lean body mass (LBM) and total body water (TBW) are reported in **Table 2** and were normal for their age and physical status. The MOTS cohort had BMD and FRAX scores representative of an osteopenic population with a moderate risk of fracture. Also, both groups experienced almost an equal number of fractures. Each participant presented with osteopenia in different parts of the body (e.g. lumbar spine, total hip and femoral neck); however, these same regions are commonly affected as already discussed in the introduction. Although the bone markers, vitamin D<sub>3</sub> and CRP levels varied widely among subjects, these variations were observed in both cohorts— placebo and MSDK. Psychological evaluation suggested that all subjects had normal mental health with no significant anxiety, stress, depression or severe menopause related symptoms. Nearly 87% subjects were taking either calcium/vitamin D<sub>3</sub>, multivitamins and/or other dietary supplements. Almost all subjects were recognized as healthy sleepers with an active lifestyle. Despite using a computer-generated block randomization scheme for stratification, significant differences in serum CTx level and diastolic pressure were found between MSDK and placebo groups, where baseline values for each were higher in the MSDK vs. placebo. All other parameters did not differ between cohorts suggesting an overall and well-adjusted randomization. Baseline characteristics of the cohort, both total and stratified are illustrated in **Table 2** and graphically represented as value per individuals at **Figure 13**.

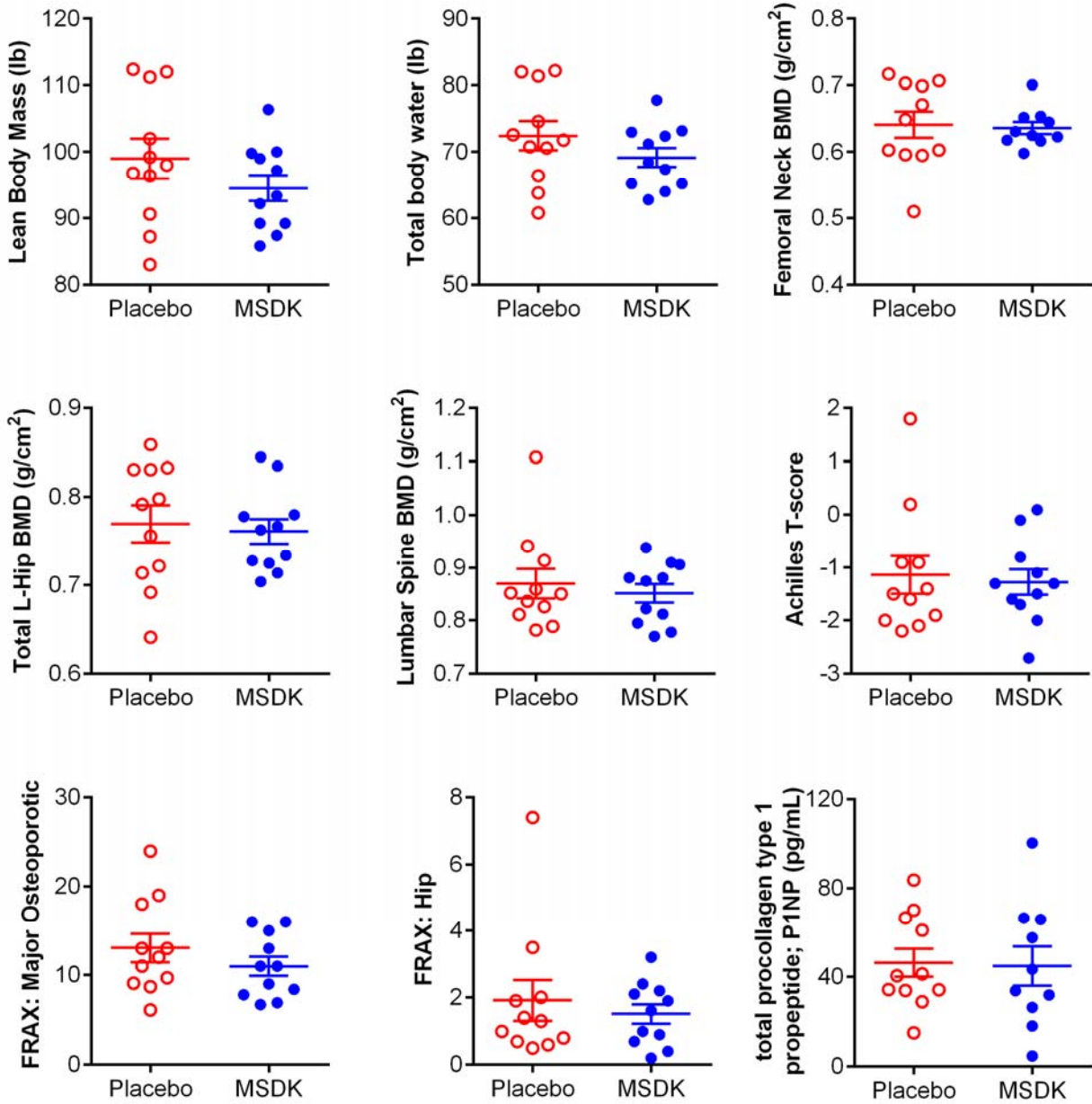
|   | Un-stratified              | Stratified                  |                             |
|---|----------------------------|-----------------------------|-----------------------------|
|   |                            | Placebo (n=11)              | MSDK (n=11)                 |
| Age   | 58.6 ± 1.12 (49-75)        | 57 ± 1.41 (49-63)           | 60 ± 1.73 (54-75)           |
| Blood pressure (BP)                               |                            |                             |                             |
| Systolic (mmHg)                                   | 118.1 ± 2.47 (100-140)     | 118.8 ± 3.29 (100-140)      | 117.4 ± 3.83 (100-140)      |
| Diastolic (mmHg)                                  | 73.4 ± 1.24 (61-80)        | 70.91 ± 1.76 (61-80)        | 75.91 ± 1.44 (64-80)*       |
| Height (cm)                                       | 166.6 ± 1.64 (154.9-182.9) | 170.2 ± 2.13 (158.8-182.9)  | 163 ± 2.06 (154.9-172.7)    |
| Weight (lb)                                       | 144.6 ± 4.83 (113.4-198)   | 147.9 ± 6.68 (121.4-177.2)  | 141.2 ± 7.16 (113.4-198)    |
| BMI (kg/m <sup>2</sup> )                          | 23.62 ± 0.7 (19.5-31.7)    | 23.16 ± 0.93 (19.6-28)      | 24.09 ± 1.08 (19.5-31.5)    |
| BMR (kcal/24h)                                    | 1319 ± 23 (1162-1543)      | 1347 ± 31.06 (1215-1509)    | 1291 ± 33.16 (1162-1543)    |
| Fat Mass (lb)                                     | 47.84 ± 3.51 (24.2-91.6)   | 48.96 ± 4.39 (33.2-75.6)    | 46.71 ± 5.67 (24.2-91.6)    |
| Fat (%)   | 32.3 ± 1.32 (21.4-46.3)    | 32.52 ± 1.59 (25.3-43.2)    | 32.08 ± 2.18 (21.4-46.3)    |
| Lean Body Mass (lb)                               | 96.75 ± 1.81 (83-112.4)    | 98.98 ± 2.99 (83-112.4)     | 94.51 ± 1.94 (85.8-106.4)   |
| Total Body Water (lb)                             | 70.8 ± 1.32 (60.8-82.2)    | 72.45 ± 2.18 (60.8-82.2)    | 69.14 ± 1.42 (62.8-77.8)    |
| BMD (DXA)   |                            |                             |                             |
| Femoral Neck (g/cm <sup>2</sup> )                 | 0.641 ± 0.01 (0.511-0.717) | 0.641 ± 0.02 (0.511-0.717)  | 0.636 ± 0.01 (0.598-0.701)  |
| Total Left Hip (g/cm <sup>2</sup> )               | 0.768 ± 0.01 (0.641-0.859) | 0.769 ± 0.02 (0.641-0.859)  | 0.761 ± 0.01 (0.704-0.845)  |
| Lumbar Spine (g/cm <sup>2</sup> )                 | 0.865 ± 0.02 (0.771-1.108) | 0.869 ± 0.03 (0.782-1.108)  | 0.852 ± 0.02 (0.771-0.938)  |
| Achilles heel T-score                             | -1.2 ± 0.21 [(-2.7)-(1.8)] | -1.14 ± 0.36 [(-2.2)-(1.8)] | -1.27 ± 0.24 [(-2.7)-(0.1)] |
| Previous fractures (1-2)                          | 8 (36%)                    | 4 (36%)                     | 4 (36%)                     |
| FRAX: major Osteoporotic                          | 12.02 ± 0.96 (6.1-24)      | 13.05 ± 1.59 (6.1-24)       | 10.98 ± 1.07 (6.7-16)       |
| FRAX: Hip   | 1.71 ± 0.33 (0.2-7.4)      | 1.92 ± 0.61 (0.5-7.4)       | 1.51 ± 0.28 (0.2-3.2)       |
| Bone markers                                      |                            |                             |                             |
| P1NP (pg/mL)                                      | 45.71 ± 5.24 (4.75-100.5)  | 46.41 ± 6.31 (15.23-83.87)  | 44.95 ± 8.91 (4.75-100.5)   |
| Osteocalcin (ng/mL)                               | 24.68 ± 1.95 (9.78-52.89)  | 24.55 ± 2.51 (9.77-36.03)   | 24.82 ± 3.11 (16.17-52.9)   |
| CTx (ng/mL)                                       | 8.78 ± 0.85 (3.89-18.97)   | 7.08 ± 0.84 (3.89-12.31)    | 10.48 ± 1.33 (4.6-18.97)*   |
| Vitamin D <sub>3</sub> (ng/mL)                    | 16.72 ± 4.71 (0.5-100.9)   | 20.77 ± 8.77 (0.83-100.9)   | 12.68 ± 3.59 (0.5-38.61)    |
| CRP (ng/mL)                                       | 2334 ± 962.7 (124.1-17974) | 2018 ± 1132 (124.1-11705)   | 2621 ± 1573 (124.1-17974)   |
| MENQOL  |                            |                             |                             |
| Vasomotor   | 2.71 ± 0.36 (1-6)          | 2.61 ± 0.56 (1-6)           | 2.82 ± 0.47 (1-5)           |
| Physical  | 2.72 ± 0.27 (1.05-5.55)    | 2.75 ± 0.36 (1.1-5.5)       | 2.69 ± 0.42 (1.05-5.3)      |
| Psychosocial                                      | 2.61 ± 0.31 (1-6.67)       | 2.8 ± 0.53 (1-6.67)         | 2.42 ± 0.36 (1-4.5)         |
| Sexual  | 2.94 ± 0.39 (1-6.67)       | 2.97 ± 0.49 (1-5)           | 2.91 ± 0.63 (1-6.67)        |
| STAI: State                                       | 30.82 ± 2.28 (20-58)       | 28.45 ± 3.34 (20-58)        | 33.18 ± 3.11 (20-53)        |
| STAI: Trait                                       | 34.41 ± 2.25 (20-65)       | 33.82 ± 3.71 (22-65)        | 35 ± 2.75 (20-45)           |
| PSS   | 11.73 ± 1.71 (1-30)        | 11.45 ± 2.39 (1-30)         | 12 ± 2.56 (2-26)            |
| CES-D   | 6.41 ± 1.63 (0-37)         | 7.09 ± 3.09 (0-37)          | 5.73 ± 1.21 (1-13)          |
| Calcium-vitamin D <sub>3</sub> /<br>Multivitamins | 19 (86.4%)                 | 10 (90%)                    | 9 (81.8%)                   |

**Table 2: Baseline characteristics of the study cohort un-stratified and stratified by treatment**

**(MSDK and placebo).** n=11 per group and represented as mean ± S.E.M. (Range). BMI= body mass index, BMR= basal metabolic rate, P1NP= total procollagen type 1 amino-terminal propeptide, OC= osteocalcin, CTx= collagen type I c-telopeptide, MENQOL= menopause specific quality of life, STAI= state and trait anxiety inventory, PSS= perceived stress scale, CES-D= center for epidemiologic studies- depression. \**p* < 0.05 vs. placebo.

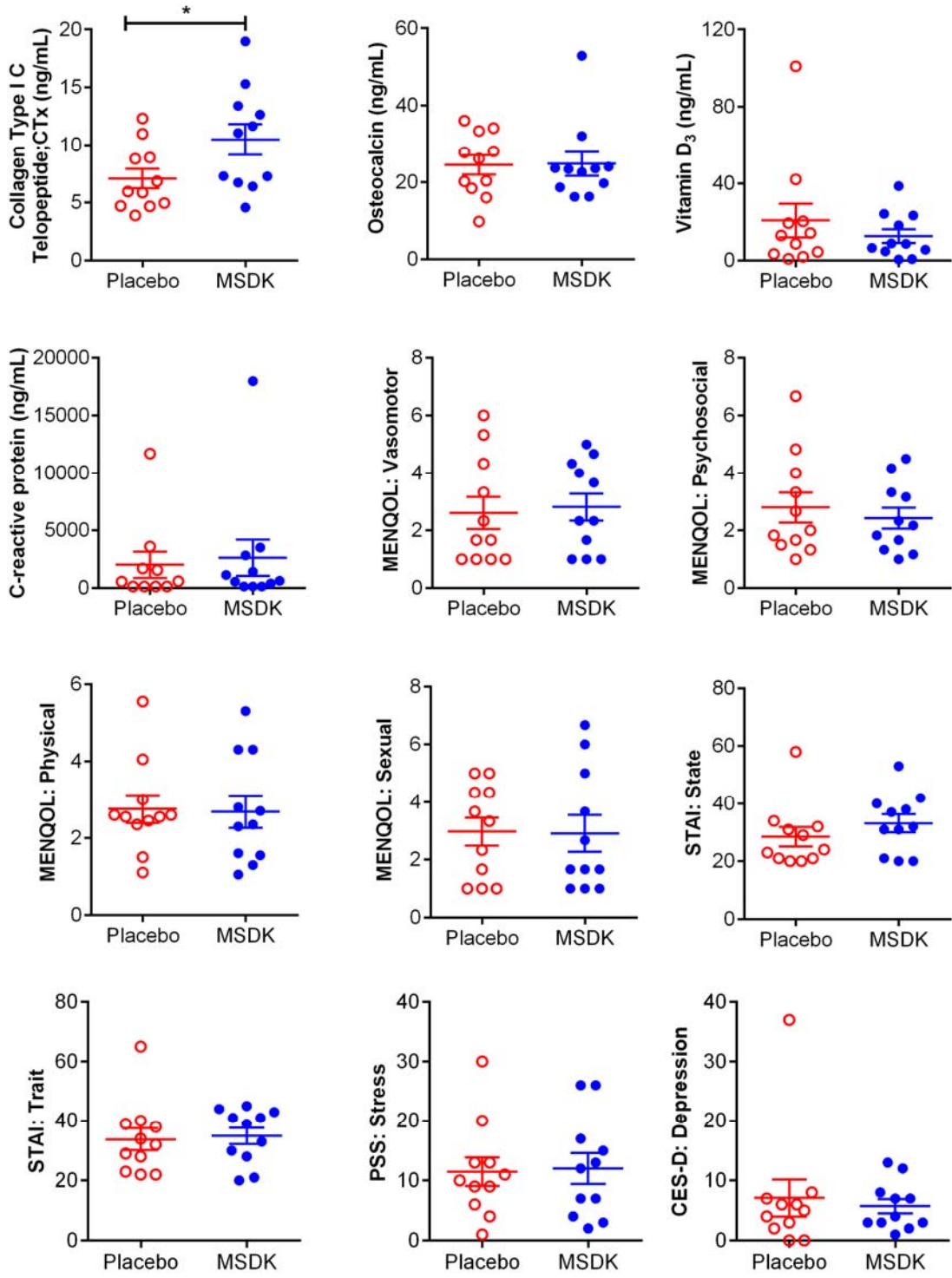


**Figure 13. Baseline characteristics of the study cohort stratified by treatment (MSDK and Placebo).** Each dot in the scatter plot represents the baseline parameters (age, blood pressure, height, weight, body mass index, basal metabolic rate, fat mass and fat%) for placebo (open circle, red) and treatment (closed circle, blue). \* $p \leq 0.05$  vs. placebo.



**Figure 13 (continued). Baseline characteristics of the study cohort stratified by treatment (MSDK and Placebo).** Each dot in the scatter plot represents the baseline parameters (lean body mass, total body water, BMD, Achilles T-score, FRAX and P1NP) for placebo (open circle, red) and treatment (closed circle, blue). \* $p \leq 0.05$  vs. placebo.





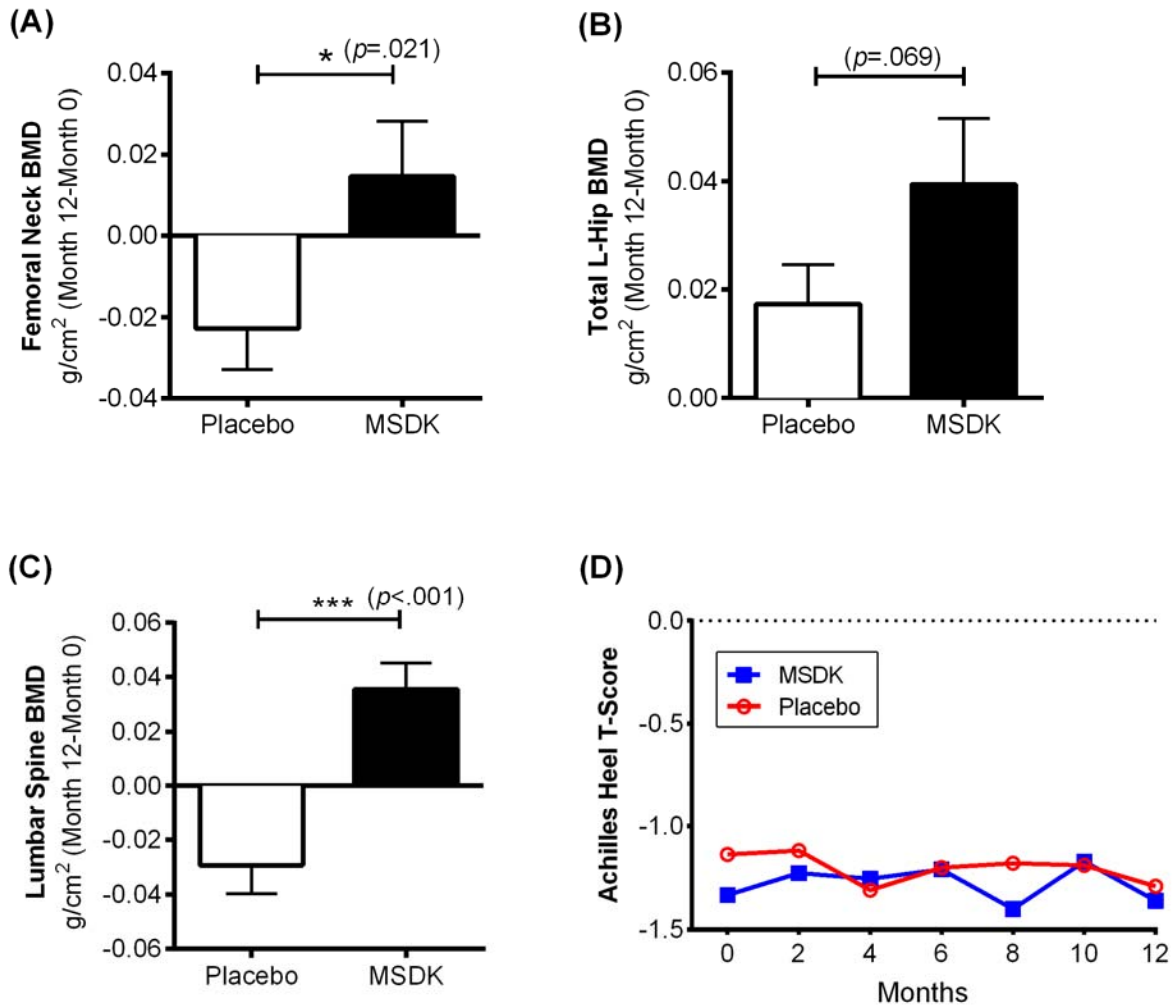
**Figure 13 (continued). Baseline characteristics of the study cohort stratified by treatment (MSDK and Placebo).** Each dot in the scatter plot represents the baseline parameters (bone markers, vitamin D<sub>3</sub>, CRP, menopausal symptoms, anxiety, stress and depression) for placebo

(open circle, red) and treatment (closed circle, blue). \* $p \leq 0.05$  vs. placebo.

### 2.2.3. MSDK improved bone health in postmenopausal women

To assess the effect of MSDK on bone health in MOTS cohort, three different parameters of bone health were monitored over the course of the one-year study— BMD via DXA and heel ultrasonography (Figure 14), fracture risk probability via FRAX (Figure 15) and serum bone markers via ELISA (Figure 16).

***MSDK increased bone density.*** As shown in **Figure 14**, the mean change in BMD ( $\text{g}/\text{cm}^2$ ) from baseline to month 12 in the left femoral neck, total left hip, lumbar spine and heel, as revealed by DXA scanning, increased in women taking MSDK. Specifically, in the femoral neck, women taking MSDK had an average BMD change of +0.015 (2.2%), whereas participants in the placebo group had an average BMD change of -0.023 (-3.6%) over one year. Data analysis showed significant differences between groups ( $p = 0.021$ ) (**Figure 14**). In the total left hip, the average BMD change was +0.039 (5.03%) for women taking MSDK and +0.017 (2.2%) for placebo. Even though analyses showed no significant difference in BMD between groups in this area, a trend ( $p = 0.069$ ) for an increase in total left hip BMD in the MSDK group was observed (**Figure 14**). Women taking MSDK showed the most significant increase in BMD in the lumbar spine (L1-L4) area ( $p < 0.001$  vs. placebo) (**Figure 14**). Specifically, the mean BMD changes in lumbar spine for MSDK was +0.035 (4.3%) and for placebo was -0.029 (-3.2%). Corresponding mean ( $\pm$  S.E.M) changes in bone density T-scores following one-year of MSDK treatment are shown in **Table 3** and their bone density T-scores are shown in **Appendix V**. Bimonthly investigation of heel bone density T-scores revealed by Achilles ultrasonography showed high variability in T-scores within groups and did not significantly differ between MSDK and placebo (**Figure 14** and **Table 3**). These findings suggest that this mode of bone measurement is relatively less reliable.

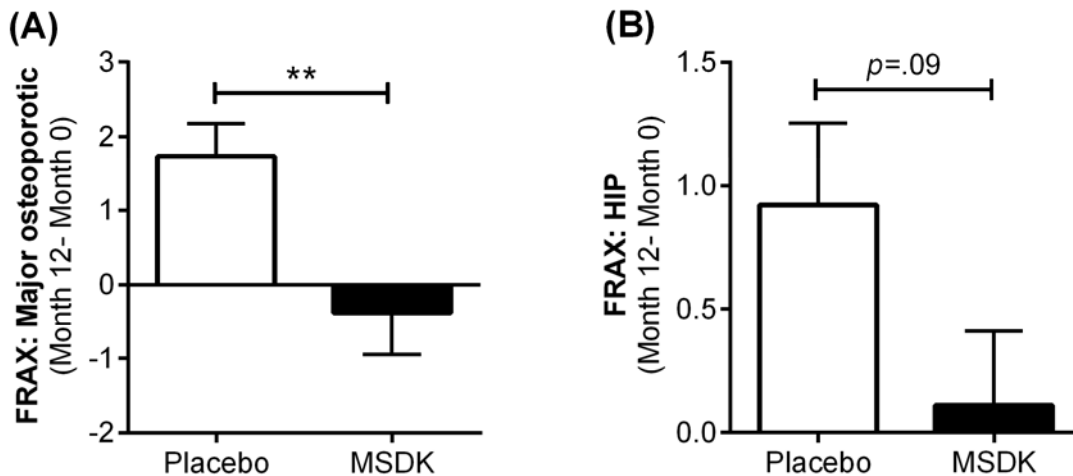


**Figure 14. Treatment effects on bone mineral density (BMD) in placebo and MSDK groups.**

Bone mineral density (g/cm<sup>2</sup>) was assessed at baseline and month 12 via DXA. Each bar in the column graph represents the mean ( $\pm$  S.E.M.) change in BMD (g/cm<sup>2</sup>) from baseline to month 12 in the **(A)** left femoral neck, **(B)** total left hip and **(C)** lumbar spine (L1-L4) area, respectively for placebo (open bar) and MSDK (closed bars); n=11 per group. \* $p \leq 0.05$  and \*\*\* $p \leq 0.001$  vs. placebo, unpaired one tailed  $t$ -test with Welch's correction. **(D)** Heel bone density T-score was measured bi-monthly using Achilles ultrasonometer. Each point in the line graph represents the mean ( $\pm$  S.E.M.) change in heel bone density T-scores at months 0, 2, 4, 6, 8, 10 and 12 respectively, for placebo (open circle, red) and MSDK (closed box, blue); n=11 per group.

Longitudinal analysis for repeated measures using a generalized linear mixed model (GLMM) approach, considering groups and times as fixed effects and subjects nested within the groups as random.

**MSDK decreased fracture risk probability.** FRAX<sup>®</sup> assessment data, as shown in **Figure 15A** and **Table 3**, indicated a significant and beneficial effect of MSDK treatment on the 10-year probability of major osteoporotic fracture risks. Specifically, one-year MSDK treatment significantly reduced the major vertebral and non-vertebral osteoporotic fracture risk by 6.48% from baseline compared to the 10.82% increase in placebo ( $p = 0.008$ ). A trend towards a decrease in hip fracture risk was observed in women taking MSDK ( $p = 0.09$  vs. placebo) (**Figure 15B**).



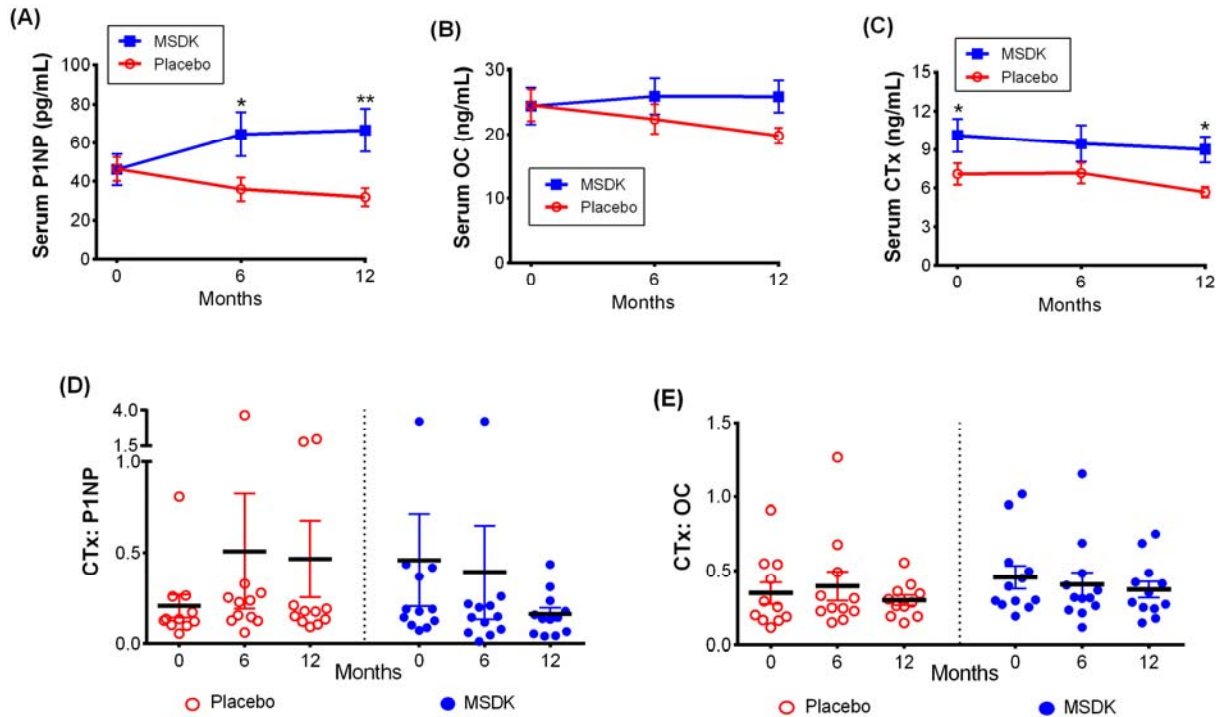
**Figure 15. Treatment effects on fracture risk probability (FRAX) in placebo and MSDK groups.**

Probability of (A) major osteoporotic and (B) hip fracture risks were measured via FRAX. Each bar in the column graph represents the mean ( $\pm$  S.E.M.) change in FRAX scores from baseline to month 12 for placebo (open bar) and MSDK (closed bars);  $n = 11$  per group. \*\* $p \leq 0.01$  vs. placebo, unpaired two tailed  $t$ -test with Welch's correction.



**MSDK decreased bone marker turnover.** To identify potential mechanisms underlying MSDK-mediated increases in bone density, serum bone formation markers, total procollagen type 1 amino-terminal propeptide (P1NP) and osteocalcin (OC) and the bone resorption marker, collagen type I cross-linked telopeptide (CTx) were analyzed at baseline, month 6 and month 12 (**Figure 16** and **Table 3**). MSDK treatment significantly increased serum P1NP levels at month 6 ( $p = 0.023$ ) and month 12 ( $p = 0.004$ ) compared to placebo (**Figure 16A** and **Table 3**). Serum P1NP levels varied widely within each group, ranging from 2.75 to 48.14 pg/mL (average:  $31.76 \pm 4.75$ ) in placebo and 25.04 to 151.9 pg/mL (average:  $66.62 \pm 11.09$ ) in MSDK at month 12. The analysis of P1NP over time revealed that the mean increase in P1NP levels in the MSDK group occurred primarily in the first six months of treatment (data shown in **Appendix VIA**). Serum osteocalcin (OC) levels did not significantly differ between groups at any time point; however, a gradual decrease in OC levels was observed in the placebo group, whereas levels remained steady in the MSDK group ( $p=0.071$  vs. placebo at month 12). At month 12, the average level of OC in women taking placebo was  $25.88 (\pm 2.5)$  ng/mL and  $19.78 (\pm 1.21)$  ng/mL in women taking MSDK, respectively (**Figure 16B** and **Table 3**). Even though the serum bone resorption marker, CTx levels were significantly higher in the MSDK group at baseline compared to placebo, it remained steady throughout the study, suggesting that MSDK either had no intrinsic effects on CTx or the dose of MSDK was not high enough to compensate for the higher baseline CTx levels in this group. Average CTx levels at month 12 in women taking MSDK was  $8.99 (\pm 1.01)$  ng/mL and in women taking placebo was  $5.65 (\pm 0.42)$  ng/mL, respectively (**Figure 16C** and **Table 3**). The analysis of OC and CTx over time are also portrayed graphically in **Appendix VIB** and **C**, respectively. Since osteoclast activity is tightly coupled to osteoblast activity ([Matsuo and Irie 2008](#)), bone marker turnover was assessed by calculating the ratio of bone resorption (CTx) to bone formation (P1NP or OC). Despite having

higher baseline CTx level in the MSDK group (vs. placebo), the ratio of CTx:P1NP trended towards a time-dependent decrease in women taking MSDK compared to the time-dependent increase observed in women taking placebo (**Figure 16**). Although not significant, a similar trend towards a time-dependent decrease in the ratio of CTx:OC was observed in women taking MSDK, which was not observed in women taking placebo (**Figure 16**).



**Figure 16. Treatment effects on serum bone markers turnover in placebo and MSDK groups.**

Bone turnover markers were assessed at months 0, 6 and 12 using ELISA. Each point in the line graph represents the mean ( $\pm$  S.E.M.) concentration of bone formation markers **(A)** total procollagen type 1 amino-terminal propeptide (P1NP) and **(B)** osteocalcin (OC; both intact and N-terminal mid-fragments); and **(C)** bone resorption marker collagen type I c-telopeptide (CTx) at months 0, 6, and 12, respectively for placebo (open circle, red) and MSDK (closed box, blue);  $n=11$  per group. Each point in the scatter plot represents the ratio of **(D)** CTx: P1NP and **(E)** CTx: OC for each study subject, respectively at specific time point, where the solid line indicates the

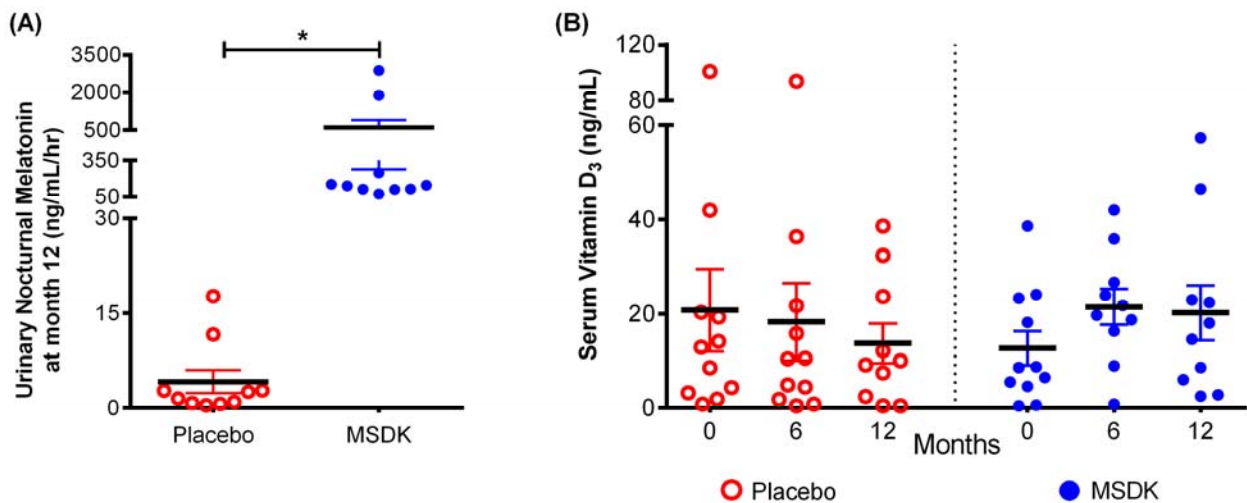
mean ( $\pm$  S.E.M.) for each group.  $*p \leq 0.05$  and  $**p \leq 0.01$  vs. placebo at similar time points. Longitudinal analysis for repeated measures using a generalized linear mixed model (GLMM) approach, considering groups and times as fixed effects and subjects nested within the groups as random.

In summary, one-year supplementation with MSDK significantly increased bone density in the left femoral neck and lumbar spine, with a trend towards an increase in the left total hip. These increases in bone density were associated with a reduction in major osteoporotic fracture risk probability. No such effect of MSDK on heel bone density T-score and hip fracture risk were observed. MSDK treatment also showed a decrease in bone marker turnover (i.e. CTx:P1NP) over the course of one year primarily through an increase in the bone formation marker, P1NP. Mean ( $\pm$  S.E.M) changes of bone density T-score, FRAX score, and bone marker per group in one year are stated in **Table 3**.

#### **2.2.4. One-year MSDK supplementation increased nocturnal melatonin levels, but did not change serum vitamin D<sub>3</sub> levels in postmenopausal women**

**Figure 17A** illustrates the nocturnal urinary melatonin sulfate level assessed at month 12. Women taking MSDK had significantly higher levels of urinary melatonin-sulfate levels compared to placebo ( $p= 0.0463$ ). Urinary melatonin-sulfate levels in the placebo group ranged from 0.43 to 17.69 ng/mL/hr (average:  $4.19 \pm 1.83$ ). Despite administering an equal nightly dose of melatonin-containing supplements for one year, participants in the MSDK group showed a wide variation in their nighttime melatonin-sulfate levels ranging from 73.13 to 2883 ng/mL/hr (average:  $586.4 \pm 309.4$ ). Melatonin levels were found to positively correlate with the annual changes in lumbar spine BMD ( $p=0.029$ ; correlation co-efficient,  $r= 0.487$ ;  $R^2= 0.24$ ; 95% CI= 0.0566 to 0.7648), supporting the requirement of daily melatonin for maintaining bone density.

Participants' serum vitamin D<sub>3</sub> levels were assessed at month 0, 6 and 12 (**Figure 17B**). Unlike melatonin, vitamin D<sub>3</sub> levels did not differ between groups at baseline and over each time point even though participants in either group could take an additional 1000IU of vitamin D<sub>3</sub> supplement for ethical reasons. A wide variability in serum vitamin D<sub>3</sub> levels occurred in both groups ranging from 2.54 to 57.32 ng/mL (average: 20.19 ± 5.84) in MSDK and from 0.5 to 38.59 ng/mL (average: 13.71 ± 4.24) in placebo at month 12. Mean changes in vitamin D<sub>3</sub> levels over a year per group are shown in **Table 3**. Correlations between vitamin D<sub>3</sub> levels and bone resorption were performed since it was shown that low vitamin D<sub>3</sub> levels are associated with high CTx levels ([Napoli, Strollo et al. 2014](#)). Similar to past-published studies, serum D<sub>3</sub> levels in MOTs negatively correlated with the CTx levels ( $p=0.024$ ; correlation co-efficient,  $r= -0.5011$ ;  $R^2= 0.25$ ; 95% CI= -0.7723 to -0.0752).

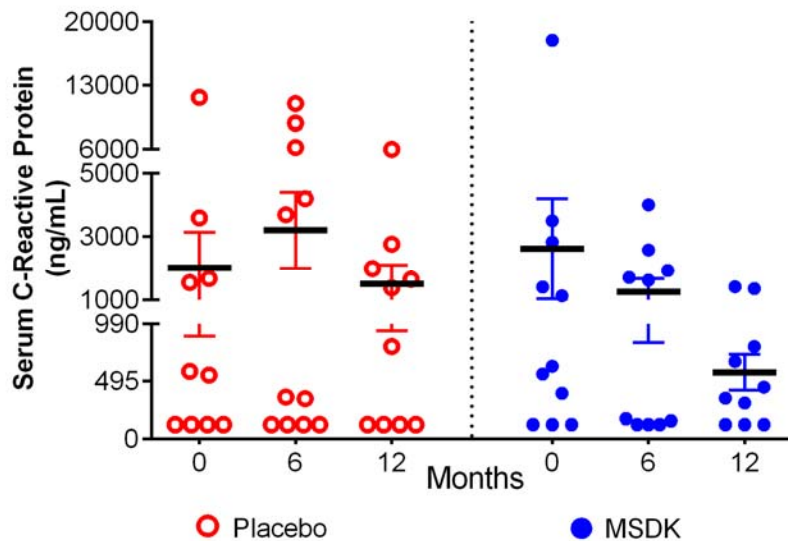


**Figure 17. Treatment effects on urinary nocturnal melatonin and serum vitamin D<sub>3</sub> in placebo and MSDK groups.** (A) Nocturnal hourly melatonin secretion in urine was measured at month 12 via ELISA. Each point in the scatter plot represents an individual's urine melatonin-sulfate level, in placebo (open circle, red) and MSDK (closed circle, blue); (n=10 per group). Solid line indicates mean (± S.E.M.) concentration per group. \* $p \leq 0.05$  vs. placebo at month 12; unpaired one-tailed

*t*-test with Welch's correction. **(B)** Serum levels of Vitamin D<sub>3</sub> was measured at months 0, 6 and 12 via ELISA. Each point in the scatter plot represents the concentration for a single subject at a specific time point, in placebo (open circle, red) and MSDK (closed circle, blue); (n=11 per group). Solid line indicates mean ( $\pm$  S.E.M.) concentration per group. \* $p \leq 0.05$  vs. placebo at similar time point; Longitudinal analysis for repeated measures using generalized linear mixed model (GLMM) approach, considering groups and times as fixed effects and subjects nested within the groups as random.

#### **2.2.5. MSDK had possible declination effect on serum C-reactive protein (CRP)**

The inflammatory marker, C-reactive protein (CRP), was measured in serum at months 0, 6 and 12 via ELISA. CRP levels varied widely within and between groups ranging from 124 to 1422 ng/mL (mean  $573.1 \pm 154.7$ ) in MSDK and from 124.1 to 6023 ng/mL (mean  $1513 \pm 581.2$ ) in placebo at month 12. Mean changes in serum CRP levels over the course of the study per group are shown in **Table 3**. As shown in **Figure 18**, serum CRP levels did not differ significantly within or between groups; however, levels were lower in women taking MSDK at month 12— an effect not seen in placebo. These findings may indicate a favorable effect of MSDK on inflammatory status.



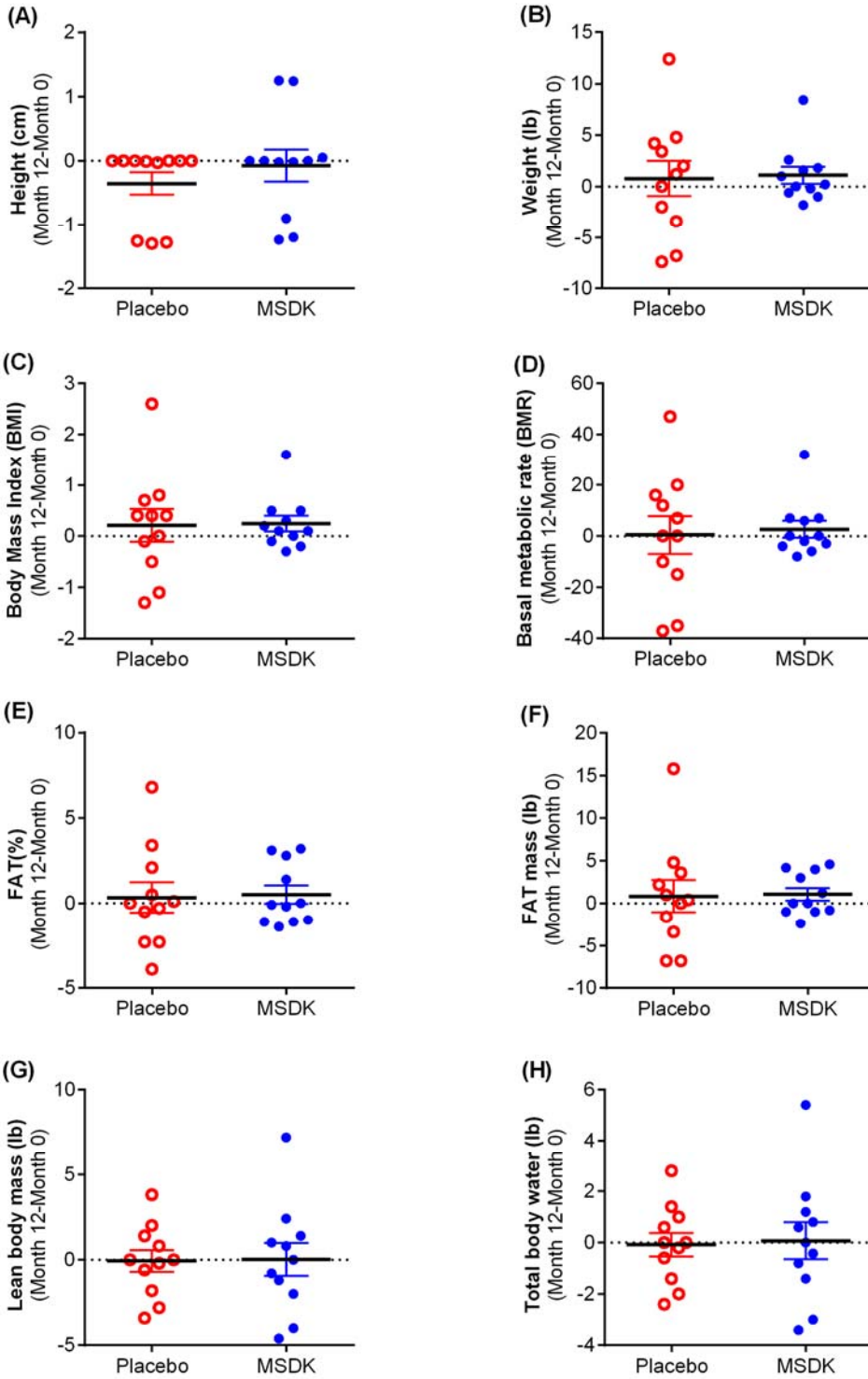
**Figure 18. Treatment effects on serum C-reactive protein (CRP) in placebo and MSDK groups.**

Serum CRP levels were measured at months 0, 6 and 12 via ELISA. Each point in the scatter plot represents the concentration for a single subject at a specific time point, in placebo (open circle, red) and MSDK (closed circle, blue); n=11 per group. Solid line indicates mean ( $\pm$  S.E.M.) concentration per group. \* $p \leq 0.05$  vs. placebo at similar time point; Longitudinal analysis for repeated measures using generalized linear mixed model (GLMM) approach, considering groups and times as fixed effects and subjects nested within the groups as random.

### **2.2.6. MSDK did not affect morphometric parameters and blood pressure, favoring bone health in postmenopausal women**

Morphometric changes from baseline (month 0) to month 12, as illustrated in **Figure 19** and **Table 3**, revealed that MSDK treatment did not improve or worsen any of the parameters of body composition. However, average height (cm) losses in the MSDK group were low (0.08) compared to placebo (0.35 cm) ( $p= 0.38$ ). Moreover, variance analysis via F-test showed that participants in the MSDK group had less fluctuation in their weight change ( $F=4.248$ ,  $DFn=10$ ;  $p= 0.032$ ), BMI change ( $F=4.112$ ,  $DFn=10$ ;  $p= 0.036$ ), BMR change ( $F=4.936$ ,  $DFn=10$ ;  $p= 0.019$ )

and fat mass change ( $F=6.409$ ,  $DFn=10$ ;  $p=0.007$ ) from baseline to month 12 compared to placebo. The lack of weight variation over time in women taking MSDK may produce favorable effects on their bone mass ([Labouesse, Gertz et al. 2014](#)). An increase in lean body mass and total body water may have occurred in response to MSDK, whereas an opposite trend was observed for placebo. Mean changes in lean body mass in MSDK and placebo groups were 0.02 and -0.07, respectively. Similarly, mean changes in total body water in MSDK and placebo groups were 0.02 and -0.07, respectively.

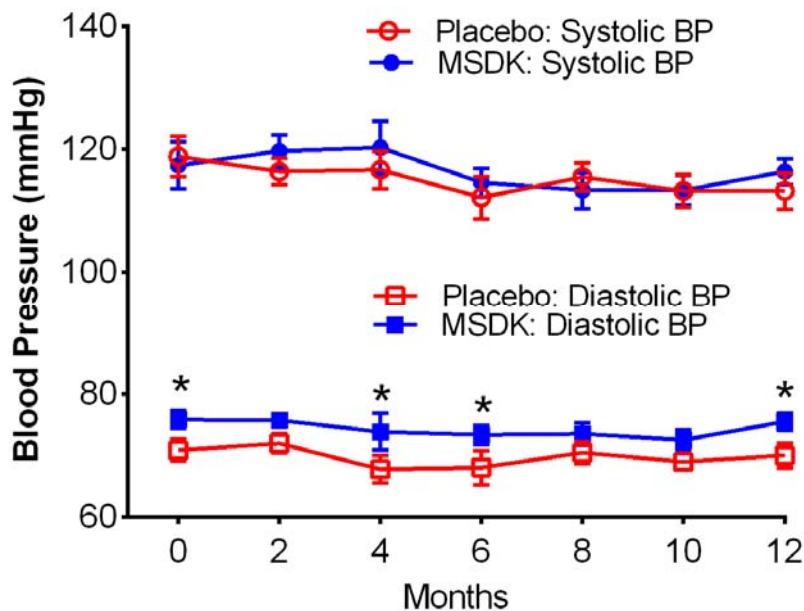


**Figure 19. Treatment effects on body composition in placebo and MSDK groups.** Body compositions were measured at months 0 and 12 using TANITA™ body composition analyzer. Each point in the scatter plot represents an individual’s change in a specific morphometric



parameter; placebo (open circle, red) and MSDK (closed circle, blue); n=11 per group. Solid line indicates mean ( $\pm$  S.E.M.) change per group from month 0 to month 12. \* $p \leq 0.05$ , \*\* $p \leq 0.01$  and \*\*\* $p \leq 0.001$  vs. placebo, unpaired two tailed  $t$ -test with Welch's correction.

Bimonthly assessments of blood pressure, as shown in **Figure 20**, demonstrated that both systolic and diastolic levels remained in the normal range throughout the study even though baseline diastolic BP in the MSDK group was significantly higher compared to placebo. Mean ( $\pm$  S.E.M) changes in blood pressure following one-year treatment can be found in **Table 3**.



**Figure 20. Treatment effects on blood pressure in placebo and MSDK groups.** Blood pressure was measured bi-monthly. Each point in the line graph represents the mean ( $\pm$  S.E.M.) blood pressure at a specific time point for placebo and MSDK (n=11 per group). (Systolic BP: solid line graph; placebo= open circle, red; MSDK= closed circle, blue. Diastolic BP: break line graph; placebo= open box, red; MSDK= close box, blue). \* $p \leq 0.05$  vs. placebo at similar time points. Longitudinal analysis for repeated measures using a generalized linear mixed model (GLMM)

approach, considering groups and times as fixed effects and subjects nested within the groups as random.

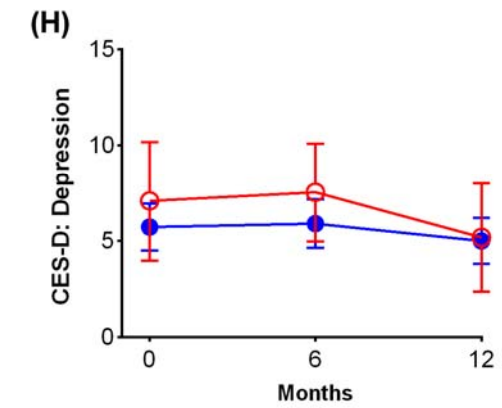
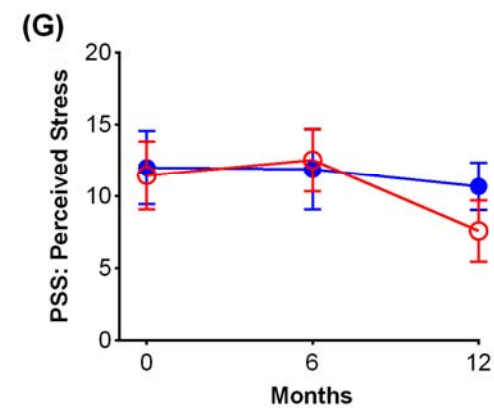
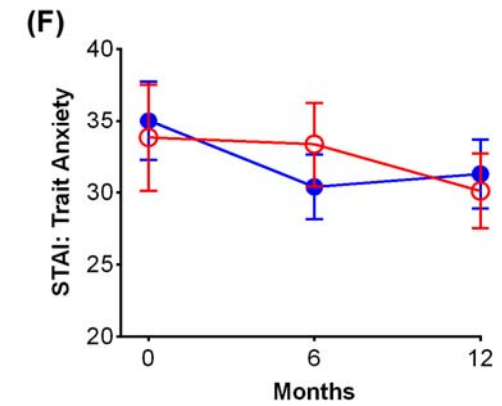
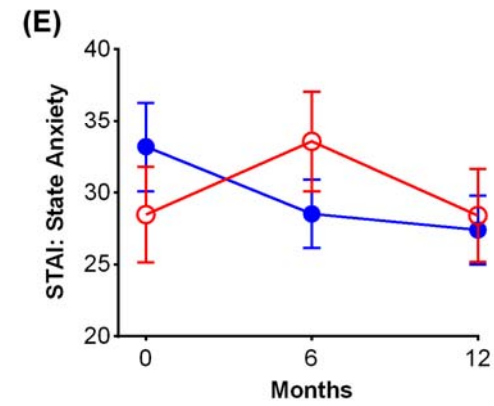
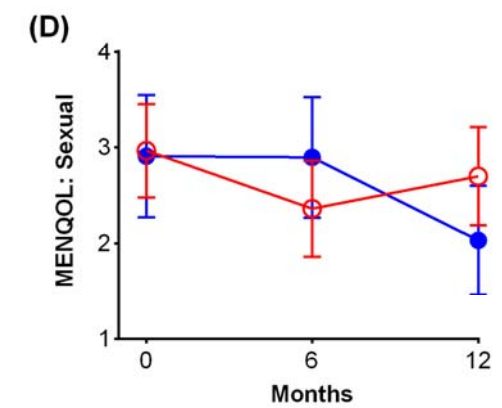
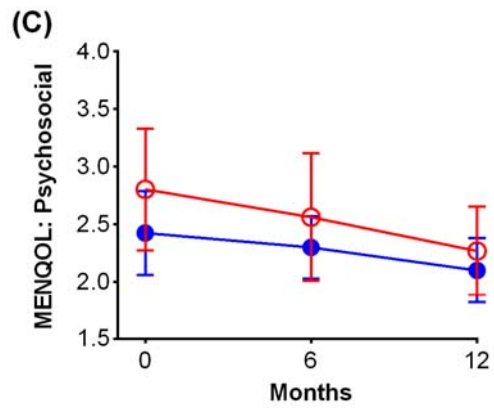
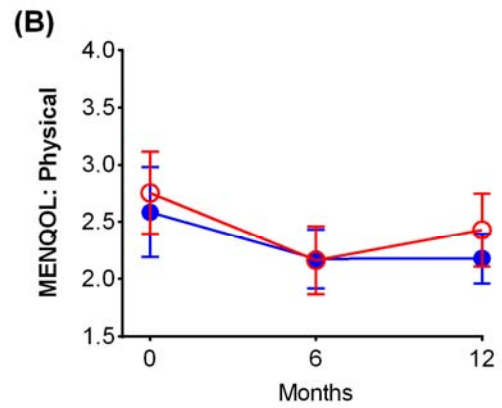
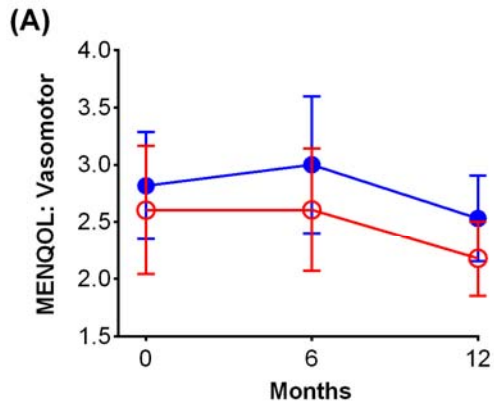
### **2.2.7. MSDK had no worsening effect on psychometric parameters in postmenopausal women**

To determine if MSDK treatment impacted on our cohort's menopausal quality of life, the validated MENQOL-Intervention questionnaire was administered at baseline, month 6 and month 12. As illustrated in **Figure 21(A-D)**, MSDK treatment showed no change in the vasomotor, physical, psychosocial and sexual domain scores compared to placebo. However, MENQOL scores in all domains were lower in both groups throughout the study suggesting that participation in the MOTS produced positive effects. Interestingly, after six months of treatment, sexual domain scores began to move in opposite directions for each group where a trend towards an improvement was observed in women taking MSDK and a trend towards a worsening was observed in placebo (**Figure 21D**). Participants' serum vitamin D<sub>3</sub> levels were found to positively correlate with their MENQOL vasomotor domain scores ( $p=0.033$ ; correlation co-efficient,  $r= 0.479$ ;  $R^2= 0.23$ ; 95% CI= 0.0459 to 0.7602). In addition, a negative correlation was observed between participants' age and annual changes in MENQOL psychosocial domain scores ( $p=0.014$ ; correlation co-efficient,  $r= -0.516$ ;  $R^2= 0.27$ ; 95% CI= -0.7704 to -0.1213).

To determine if MSDK impacted on our cohort's anxiety levels, the validated Spielberger's State-Trait Anxiety Inventory (STAI) questionnaire was administered at baseline, month 6 and month 12. Analysis showed no significant difference in the state and trait anxiety levels between MSDK and placebo (**Figure 21E and F**). At month 12, the average scores for state anxiety in MSDK and placebo groups were 27.4 and 28.4, respectively; whereas the average scores for trait anxiety was 31.3 in MSDK and 30.1 in placebo, respectively. These scores represented low state

and trait anxiety levels (range: 20-39) in almost all participants.

To determine if MSDK impacted on our cohort's perceived stress and depression status, the validated Perceived Stress Scale (PSS) and Center of the Epidemiological Study of Depression (CES-D) questionnaires were administered, respectively, at baseline, month 6 and month 12 and revealed no significant difference between groups (**Figure 21G and H**). Average PSS scores at month 12 were 10.7 in MSDK and 7.6 in placebo at month 12, reflecting a low stress level (score < 20) in study population. Similarly, the average CES-D scores in MSDK and placebo groups were 5 and 5.2, respectively, suggesting the absence of depressive symptomatology (score <16) in the study population.



**Figure 21. Treatment effects on the menopause quality of life, anxiety, stress and depression in placebo and MSDK groups.** Psychological assessments were carried out at months 0, 6 and 12 using validated questionnaires. Each point in the line graph represents the mean ( $\pm$  S.E.M.) score for (A) MENQOL vasomotor, (B) MENQOL physical, (C) MENQOL psychosocial, (D) MENQOL sexual, (E) STAI: State anxiety, (F) STAI: Trait anxiety, (G) PSS: Perceived stress scores and (H) CES-Depression at specific time point, respectively for placebo (open circle, red) and MSDK (close circle, blue); n=11 per group.  $*p \leq 0.05$  vs. placebo at similar time point. Longitudinal analysis for repeated measures using generalized linear mixed model (GLMM) approach, considering groups and times as fixed effects and subjects nested within the groups as random.

In summary, MSDK treatment did not significantly improve or worsen the symptoms associated with menopause, anxiety, stress, and depression. All participants remained in the normal range for all psychological domains throughout the study. Corresponding mean ( $\pm$  S.E.M) change per year of psychometric parameters are shown in **Table 3**.

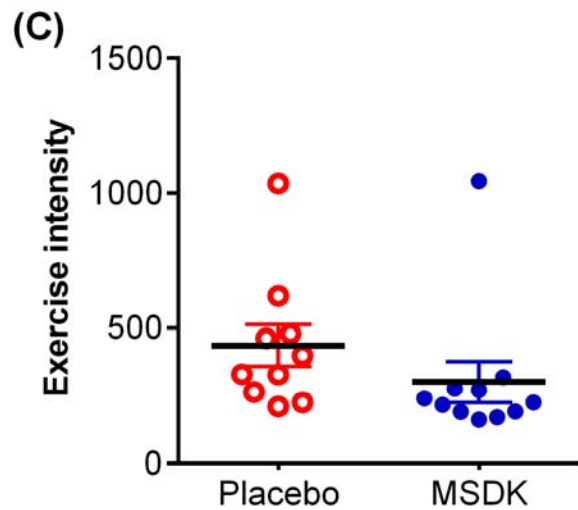
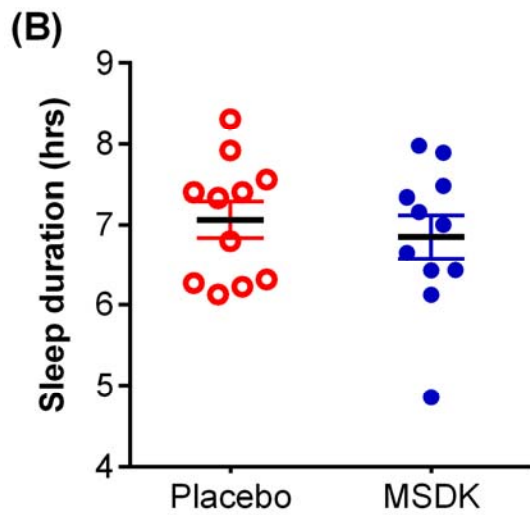
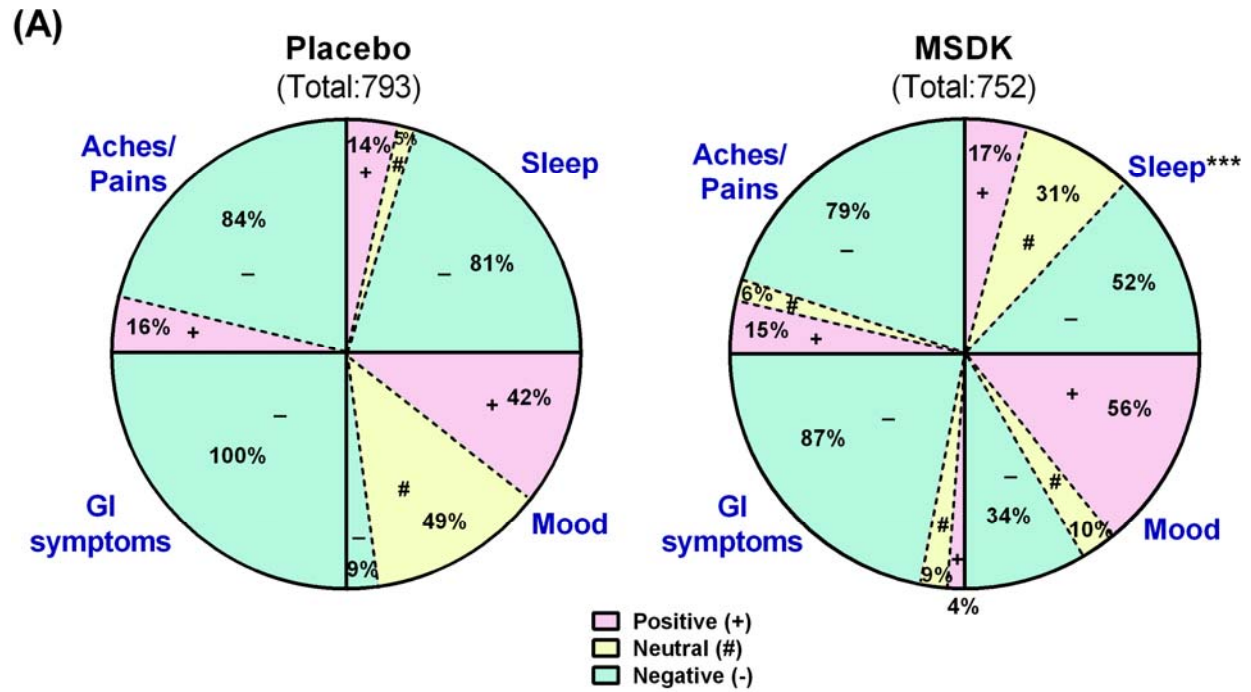
|                                | Placebo |          |                        | MSDK    |          |                           |
|--------------------------------|---------|----------|------------------------|---------|----------|---------------------------|
|                                | Month 0 | Month 12 | Change                 | Month 0 | Month 12 | Change                    |
| <b>T-Scores</b>                |         |          |                        |         |          |                           |
| Femoral Neck                   | -1.87   | -2.13    | <b>-0.20 (0.09)</b>    | -1.88   | -1.74    | <b>0.13 (0.12) *</b>      |
| Total Left Hip                 | -1.42   | -1.33    | 0.16 (0.06)            | -1.37   | -1.02    | 0.35 (0.09)               |
| Lumber Spine (L1-L4)           | -1.61   | -1.81    | <b>-0.25 (0.09)</b>    | -1.70   | -1.39    | <b>0.31 (0.09) ***</b>    |
| Achilles (heel)                | -1.14   | -1.29    | -0.18 (0.13)           | -1.27   | -1.36    | 0.09 (0.11)               |
| FRAX: Major Osteoporotic       | 13.05   | 14.79    | <b>1.73 (0.44)</b>     | 10.98   | 10.6     | <b>-0.38 (0.56) **</b>    |
| FRAX: Hip                      | 1.92    | 2.84     | 0.92 (0.33)            | 1.51    | 1.45     | 0.11 (0.31)               |
| P1NP (pg/mL)                   | 46.41   | 31.76    | <b>-14.65 (± 4.81)</b> | 44.95   | 66.62    | <b>21.32 (± 5.66) ***</b> |
| OC (ng/mL)                     | 24.55   | 19.78    | -4.76 (± 1.92)         | 24.82   | 25.88    | -0.5811 (± 1.41)          |
| CTx (ng/mL)                    | 7.08    | 5.65     | -1.43 (± 0.72)         | 10.48   | 8.99     | -1.51 (± 0.57)            |
| Vitamin D <sub>3</sub> (ng/mL) | 20.77   | 20.19    | -7.06 (± 7.78)         | 12.68   | 13.71    | 7.51 (± 4.73)             |
| CRP (ng/mL)                    | 2017.9  | 1513     | <b>-658.5 (± 1238)</b> | 2620.9  | 573.1    | <b>(-2048 ± 1548)</b>     |
| Systolic BP (mmHg)             | 118.8   | 113.2    | -5.62 (± 2.57)         | 117.4   | 116.4    | -0.95 (± 2.68)            |
| Diastolic BP (mmHg)            | 70.91   | 70.1     | -0.81 (± 1.51)         | 75.91   | 75.6     | -0.31 (± 1.44)            |
| Height (cm)                    | 170.18  | 169.82   | -0.35 (0.18)           | 163.02  | 162.95   | -0.08 (0.25)              |
| Weight (lb)                    | 147.95  | 148.71   | 0.76 (1.71)            | 141.22  | 143.31   | 1.20 (0.83)               |
| BMI (kg/m <sup>2</sup> )       | 23.16   | 23.36    | 0.21 (0.32)            | 24.09   | 24.31    | 0.24 (0.16)               |
| BMR (kcal/24h)                 | 1347.36 | 1347.82  | 0.45 (7.37)            | 1291.27 | 1293.91  | 2.64 (3.32)               |
| Fat Mass (lb)                  | 48.96   | 49.8     | 0.84 (1.88)            | 46.71   | 47.78    | 1.07 (0.74)               |
| Fat (%)                        | 32.52   | 32.85    | 0.33 (0.89)            | 32.08   | 32.59    | 0.51 (0.54)               |
| Lean Body Mass (lb)            | 98.98   | 98.9     | -0.07 (0.63)           | 94.51   | 94.53    | 0.02 (0.97)               |
| Total Body Water (lb)          | 72.45   | 72.38    | -0.07 (0.46)           | 69.14   | 69.22    | 0.07 (0.73)               |
| <b>MENQOL</b>                  |         |          |                        |         |          |                           |
| Vasomotor                      | 2.61    | 2.30     | -0.31 (0.48)           | 3.00    | 2.53     | -0.47 (0.28)              |
| Physical                       | 2.75    | 2.43     | -0.32 (0.24)           | 2.53    | 2.18     | -0.34 (0.34)              |
| Psychosocial                   | 2.80    | 2.27     | -0.53 (0.30)           | 2.22    | 2.10     | -0.12 (0.39)              |
| Sexual                         | 2.97    | 2.70     | -0.27 (0.31)           | 2.93    | 2.03     | -0.90 (0.49)              |
| STAI: State anxiety            | 28.45   | 28.4     | -0.05 (1.09)           | 32.5    | 27.4     | -5.10 (4.08)              |
| STAI: Trait anxiety            | 33.82   | 30.1     | -3.71 (1.80)           | 34.1    | 31.3     | -2.80 (3.38)              |
| PSS: Perceived stress          | 11.45   | 7.6      | -3.85 (1.18)           | 11.5    | 10.7     | -0.80 (2.03)              |
| CES-D: Depression              | 7.09    | 5.2      | -1.89 (0.86)           | 5.1     | 5        | -0.10 (1.67)              |

**Table 3: Treatment effects on bone density (T-scores), fracture risk probability (FRAX), bone marker, body composition and psychometric parameters.** Mean (± S.E.M.). P1NP= total procollagen type 1 amino-terminal propeptide; OC= osteocalcin; CTx= collagen type I c-telopeptide; CRP= c-reactive protein; BP= blood pressure; BMI= body mass index; BMR= basal metabolic rate; MENQOL= menopause specific quality of life; STAI= state and trait anxiety inventory; PSS= perceived stress scale; CES-D= center for epidemiologic studies depression. \* $p < 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$  vs. placebo (n=11 per group).

### 2.2.8. MSDK improved general well-being and compliance in postmenopausal women

Diary comments were analyzed to assess if MSDK treatment affected our cohort's general well-being. As illustrated in **Figure 22A**, a total of 752 comments were written by women taking MSDK, whereas women on placebo made 793 comments. Analysis of these comments revealed an improvement in sleep quality in women taking MSDK, as indicated by 29% more positive/neutral comments made in the MSDK group vs. placebo ( $p < .0001$ ). The relative risk ratio of a positive sleep statement for women taking MSDK was 2.5 (95% CI= 1.44 to 4.35), suggesting that the likelihood of a positive sleep statement is 150% higher in MSDK group compared to placebo. Regarding mood, the percentage of both positive and negative comments were high in MSDK group as compared to placebo. However, analysis showed no correlation between MSDK intake and mood change in this cohort. Interestingly, all (100%) comments made about GI symptoms in the placebo group were negative whereas 87% were negative in the MSDK group—a reduction of 13%. Women in the MSDK group made some positive comments perhaps indicating improvements in GI upset. Regarding general aches and pains, the percentage of negative comments made in the MSDK group was 79% compared to 84% comments recorded for placebo. Statistical analyses revealed no significant impact of MSDK on GI symptoms and general aches/pains. Most of what was reported, positive or negative, had to do with their overall general well-being reported in their daily diaries. Although the women in MSDK group experienced an improvement in sleep quality, their sleep duration remained similar to that of placebo ( $p = 0.55$ ), as determined from their diary log (**Figure 22B** and **Table 4**). The average sleep time (in hours) for those taking MSDK or placebo was 6.85h and 7.06h, respectively. As shown in **Figure 22C** and **Table 4**, analysis of exercise intensity showed no significant differences between groups ( $p=0.23$ ); however, most participants in the placebo group were involved with high intensity

exercises whereas almost all MSDK group participants were exercised with light to moderate intensity. Both groups had an equal intake of multivitamins/herbal supplements/OTC products (81.82%). Analysis of confounders revealed no significant differences between groups (Table 4).





**Figure 22. Treatment effects on participants' (A) sleep quality, mood, GI upset and general aches/pains; (B) sleep duration and (C) exercise intensity in placebo and MSDK groups.** (A) To assess the effect of MSDK on the general well-being of the MOTS cohort, total diary comments made by the participants in each group throughout the study were stratified into four categories: sleep, mood, GI upset and general aches/pains, as illustrated by the four segments in the pie diagram. Each category was sub-stratified as positive (pink), neutral (yellow) and negative (green) comments. Each portion represents the percent of total comments made under each category.  $*p \leq 0.05$  vs. placebo; two-tailed Fishers exact test for two categorical outcomes. (B, C) Sleep duration and exercise intensity was assessed from the diary log. Each point in the scatter plot represents the scores of (B) sleep duration (in hour) and (C) exercise intensity for each participant in placebo and MSDK respectively (n=11 per group); where the solid line indicates the mean ( $\pm$  S.E.M.) score per group.  $*p \leq 0.05$  vs. placebo; unpaired one tailed *t*-test with Welch's correction.

Compliance was measured from the daily diary logs as well as from bimonthly pill counting and health assessments. No incidence of adverse effects was reported from participants in either group during their bimonthly general health checkups at the Center of Pharmacy Care at Duquesne University or through their diary logs. The fact that MSDK treatment did not worsen any of the subjective measures assessed may have impacted positively on compliance, which was high in the MOTS (Placebo=90.03% and MSDK = 92.4%; **Table 4**).

|                                   | Placebo (n=11)          | MSDK (n=11)              | <i>p</i> -value |
|-----------------------------------|-------------------------|--------------------------|-----------------|
| Medication intake                 | 90.03%                  | 92.4%                    |                 |
| Sleep duration (hr)               | 7.06 ± 0.23 (6.13-8.31) | 6.85 ± 0.27 (4.85-7.97)  | 0.55            |
| Exercise intensity                | 435 ± 77.93 (209-1036)  | 300.2 ± 75.78 (161-1044) | 0.23            |
| Multivitamins/<br>OTC supplements | 81.82%                  | 81.82%                   |                 |

**Table 4: Sleep duration, exercise intensity and pill intake in placebo and MSDK groups.** Mean ( $\pm$  S.E.M.) for sleep duration and exercise intensity (n=11 per group).

### 2.3. Discussion

Our study is one of the firsts addressing the need for starting earlier intervention therapy to reverse bone loss and normalize bone density in postmenopausal women with osteopenia. This is important because studies have shown that osteopenia is the primary accountable factor for the population burden of fracture as earliest fractures predominantly arise in the osteopenic population ([Siris, Chen et al. 2004](#), [Pasco, Seeman et al. 2006](#)). Therefore, this shift to earlier intervention starting with osteopenia rather than osteoporosis may lead to the prevention of most fractures observed worldwide. To the best of our knowledge, this is also the first randomized, double-blind, placebo-controlled study focusing on the efficacy of a timed combination therapy consisting of melatonin and micronutrients (strontium citrate, vitamin D<sub>3</sub> and vitamin K<sub>2</sub>) intended to reverse osteopenia while improving compliance in postmenopausal women. The choice of supplements were all natural and over-the-counter remedies known to benefit bone.

The inclusion and exclusion criteria of this study aimed to minimize the presence of any additional positive and/or negative effects on bone, rather than the treatment effects. Avoiding alcohol intake while taking the medication was one inclusion criterion, since alcohol consumption in the evening may suppress salivary melatonin levels ([Rupp, Acebo et al. 2007](#)). Women taking

bisphosphonates were excluded even though many of them were ready to discontinue before joining the study. This is because it takes at least 6 months to 2 years after stopping bisphosphonates to clear out of the system so not to blunt strontium's effects ([Middleton, Steel et al. 2010](#)). Selective serotonin reuptake inhibitors (SSRIs) increase osteoporosis and fracture risk, which could affect one of the primary outcome measures ([Chau, Atkinson et al. 2012](#)) and therefore was one of the reasons for being an exclusion criterion. In addition, MSDK effects on depression status was a secondary outcome measure and SSRIs could have influenced that as well. Finally, concomitant use of melatonin and Zoloft (an SSRI antidepressant also known as Sertraline) has been shown to produce an adverse drug interaction exhibiting as toxic optic neuropathy due to a melatonin/dopamine imbalance in the retina ([Lehman and Johnson 1999](#)). Gastrointestinal (GI) diseases such as inflammatory bowel disease (IBS) and celiac disease could trigger osteoporosis in many ways primarily by affecting the absorption and metabolism of calcium and vitamin D<sub>3</sub> ([Katz and Weinerman 2010](#)). Women with ulcerative colitis and other severe GI disorders were not included in the study. Even though strontium ranelate is known to occasionally cause diarrhea ([Reginster, Seeman et al. 2005](#)), which may worsen IBS flare-ups in ulcerative colitis, no such data was reported for the strontium citrate at the time of the study. Women taking proton pump inhibitors (PPIs) regularly were excluded because use of PPIs is modestly associated with fractures of the spine, forearm or wrist and increased the risk of total fractures with a hazard ratio of 1.47, 1.26 and 1.25, respectively ([Gray, LaCroix et al. 2010](#)). Another study found a potential impact of PPIs on increasing the risk of hip fractures among patients already at-risk possibly by decreasing calcium absorption ([Corley, Kubo et al. 2010](#)). Participants in the MOTS were told to restrain from using PPIs while in the study. Current smokers were excluded because smoking is associated with a decrease in BMD and a prolonged history of smoking significantly

increases the risk of fractures ([Kanis, Johnell et al. 2005](#)). Hyperparathyroidism accelerates bone loss in postmenopausal women and significantly affects bone turnover by increasing bone resorption ([Guo, Thomas et al. 1996](#)). Multiple myeloma and other cancers that metastasize to bone could also adversely affect bone metabolism and bone turnover ([Simko and Paulis 2007](#)); and sleep apnea that disturbs one's circadian rhythm can trigger bone loss in women via various mechanisms including altered glucocorticoid regulation ([Chakhtoura, Nasrallah et al. 2015](#), [Maria and Witt-Enderby 2017](#)). Therefore, women with any of these diseases were excluded from the MOTS. Furthermore, COPD-related systemic inflammation, vitamin D deficiency as well as use of systemic corticosteroids can significantly contribute to bone loss ([Lehouck, Boonen et al. 2011](#)). Women having hepatic and renal impairment were excluded because these conditions could interfere with the bioavailability of orally administered components of MSDK. The total amount of calcium and vitamin D<sub>3</sub> allowed in the MOTS including calcium and vitamin D<sub>3</sub> coming from supplements and multivitamins was up to 600 mg of calcium and 1000 IU of vitamin D<sub>3</sub> per day; since these amounts were found to not interfere with strontium effects ([Reginster, Seeman et al. 2005](#)).

The primary endpoints in this study were the assessments of bone health in response to nightly MSDK supplementation compared to placebo. Bone health was examined by measuring bone density changes before and after one-year of treatment, and bone marker status every six months over one year. The one-year timeline for bone density assessment was chosen based on former bone density studies demonstrating the requirement of at least one year to observe any changes in bone density. Osteopenic women taking MSDK for one year had significant improvements in their left femoral neck and lumbar spine BMD with a trend ( $p = .069$ ) towards an increase in the total left hip BMD compared to women taking placebo. Both MSDK and placebo

group showed an improvement in their hip BMD, perhaps due to the kinetics of bone remodeling in long vs. flat bones. Vertebral and femoral neck contain mostly the trabecular bones, which goes more readily into bone remodeling and endure more wear and tear as compared to cortical bones. Therefore, changes in bone density in these areas are more pronounced ([Clarke 2008](#)). In contrast to osteoporosis, an osteopenic population is expected to demonstrate a longer change in BMD improvement in response to treatment because their BMD status was better (or denser) than the BMD status in an osteoporotic population. In keeping, our findings are consistent with respect to the magnitude of the BMD change and the tissues being most affected (i.e. spine > femoral neck > total hip) to the previous postmenopausal health studies. In these studies, it is shown that the most significant increase in BMD following one treatment with strontium citrate, vitamin D<sub>3</sub> and vitamin K<sub>2</sub> and other micronutrients occurs in the spine (6 to 8%) followed by femoral neck (4%) and total hip (3%) BMD ([Genuis and Bouchard 2012](#)) or in spine BMD following treatment with vitamin D<sub>3</sub> and vitamin K<sub>2</sub> ([Ushiroyama, Ikeda et al. 2002](#), [Kanellakis, Moschonis et al. 2012](#)). Another study with strontium ranelate demonstrates similar increases in spine bone density in 26.4% postmenopausal osteopenic women after one year, while the number increases up to 58.2% after three years. The percentage of patients with renormalized hip bone density increases up to 5.4% after one year and 19.6% after three years of treatment ([Malaise, Bruyere et al. 2007](#)). A recent randomized control trial by Amstrup *et al* demonstrates a similar outcome where 3 mg/day melatonin supplementation for one year results in a 1.4% increase in femoral neck BMD in postmenopausal osteopenic women, whereas no significant improvement occurs at other areas ([Amstrup, Sikjaer et al. 2015](#)).

Consistent with the BMD data, women taking MSDK had a lower risk for a major osteoporotic fracture with no significant effect on hip fracture, as revealed by FRAX<sup>®</sup>. Little is

known about the effect of melatonin and other micronutrients in preventing osteoporotic fractures. Strontium ranelate is found to be effective against fractures in postmenopausal population. In the Spinal Osteoporosis Therapeutic Intervention (SOTI) trial, strontium ranelate (2 g/day) shows a 49% reduction in new vertebral fractures after one year and a 41% reduction in vertebral fracture risk after three years in postmenopausal women with mean age 70, ([Meunier, Roux et al. 2004](#)). The Treatment of Peripheral Osteoporosis (TROPOS) trial demonstrates similar results with a 16% reduction in overall non-vertebral fractures after three years treatment with strontium ranelate (2 gm/day), and a 36% reduction in hip fractures for high risk subgroups ([Reginster, Seeman et al. 2005](#)). Extensive studies reveal that vitamin D<sub>3</sub> (minimum 800 IU per day), when taken with calcium, effectively reduces the risk of hip fracture in both high and low risk populations while showing inconsistent effects against vertebral fracture. These studies also demonstrate that even though vitamin D<sub>3</sub> deficiency increases fracture risk, supplementation with vitamin D alone is ineffective in fracture prevention ([Avenell, Mak et al. 2014](#)). As shown in the MOTS, vitamin D combined with other bone-tropic agents aids in reversing bone loss in an osteopenic population of women who have gone through menopause.

Bone markers are independent fracture risk predictors, mainly used to monitor treatment efficacy. The bone formation marker, P1NP, and the bone resorption marker, CTx, were assessed in this study based on the recommendations of the Bone Marker Standards Working Group, the International Osteoporosis Foundation (IOF) and the National Bone Health Alliance (NBHA) as being the gold standard reference analytes in clinical studies. Even though intact P1NP is the preferred analyte to be measured, total serum P1NP level was measured because all our participants had normal renal function ([Wheater, Elshahaly et al. 2013](#)). Serum P1NP levels significantly increased with MSDK treatment vs. placebo throughout the course of the study. Two

randomized controlled trials with strontium ranelate found a link between short term (3-month) changes in the bone formation markers (bone-specific alkaline phosphatase, BSAP and C-terminal propeptide of type I procollagen, PICP) with a prospective 3-year change in BMD ([Bruyère, Collette et al. 2010](#)). These findings suggest that an increase in the bone formation marker, P1NP, may underlie the increases in BMD in the MOTs. Another bone formation marker, OC, was measured but its level did not increase significantly with MSDK treatment. Rather, a steady level was maintained throughout the study as compared to placebo. This is consistent with the MOPS and MelaOst demonstrating that melatonin alone (3mg) did not induce serum OC or P1NP levels in perimenopausal or postmenopausal women ([Kotlarczyk, Lassila et al. 2012](#), [Amstrup, Sikjaer et al. 2015](#)).

To our knowledge, no data is yet available on the effect of strontium citrate, vitamin D<sub>3</sub> and vitamin K<sub>2</sub> on the bone marker, P1NP. However, strontium ranelate, is shown to enhance other bone formation markers (e.g. bone-specific alkaline phosphatase, BSAP; Procollagen I carboxyterminal propeptide, PICP) ([Meunier, Roux et al. 2004](#), [Bruyère, Collette et al. 2010](#)). With respect to vitamins K<sub>2</sub> and D<sub>3</sub>, vitamin K<sub>2</sub> shows enhancing effects on serum OC ([Tsukamoto, Ichise et al. 2000](#)) whereas low levels of vitamin D<sub>3</sub> (<20ng/mL) produce a suppressive effect on P1NP ([Bacon, Gamble et al. 2009](#)). Although these compounds alone have little or variable effects on bone formation markers, their combination in MSDK produced a significant inductive effect on P1NP in the present study, which is consistent with MSDK's inductive effect on BMD.

In our study, even though MSDK group had a higher CTx level at baseline, MSDK was without effect on serum CTx levels at each time point tested. It was not clear what the effect of MSDK would have on CTx levels because the studies are mixed. For example, increases in bone resorption occur in response to low vitamin D<sub>3</sub> levels ([Napoli, Strollo et al. 2014](#)) or after removal

of the melatonin source via pinealectomy as reviewed ([Maria and Witt-Enderby 2014](#)). Strontium, in the form of ranelate, produces a reducing effect on CTx ([Meunier, Roux et al. 2004](#)); however, no effect was evaluated with citrate form. Melatonin alone ([Amstrup, Sikjaer et al. 2015](#)), vitamin D<sub>3</sub> alone ([Kuchuk, van Schoor et al. 2009](#)), or vitamin K<sub>2</sub> (MK4) alone ([Kim, Na et al. 2013](#)) produces little or no effect on serum CTx levels. These mixed effects on serum OC and CTx levels may be explained by the fact that, unlike P1NP, both OC and CTx display a circadian rhythm and, particularly for CTx, it is best to use a morning fasting sample for an accurate reading ([Wheater, Elshahaly et al. 2013](#)), which was not followed in the MOTS due to technical limitations. However, in our study, circadian variation was minimized by collecting serum samples at regular time intervals (between 4pm-5:30pm) for each participant throughout the study. Interestingly, when ratios of bone resorption to bone formation (i.e., CTx:P1NP or CTx:OC) were calculated per person and then compared within and between groups, time-dependent decreases in both occurred even though the decreases for CTx:OC were less pronounced compared to CTx:P1NP.

The effect of MSDK on decreasing the ratio of CTx:P1NP or CTx:OC was primarily mediated via increasing the bone formation marker (i.e. P1NP) or keeping them at a steady level (i.e. OC), rather than by decreasing the bone resorption marker, CTx. This mechanism of MSDK to regulate bone turnover differs from the mechanisms of most current antiresorptive therapies where both formation and resorption markers decrease proportionally. The pattern of MSDK matches, in part, with what was seen for strontium ranelate or teriparatide, particularly with respect to the bone formation marker, P1NP. Strontium ranelate regulates bone marker changes in a way that would support more balanced bone remodeling, with an approximate 8% increase in bone formation and 12% decrease in bone resorption. Teriparatide, on the other hand, induces both bone formation and resorption markers ([Meunier, Roux et al. 2004](#)). ([Bruyère, Collette et al. 2010](#)). The



changes in serum P1NP is most likely due to the strontium citrate component of MSDK since strontium ranelate increases bone-specific alkaline phosphatase (BSAP) by 8.1% ([Meunier, Roux et al. 2004](#)) and in the MOPS or MelaOst RCTs, melatonin alone did not affect individual bone marker levels ([Kotlarczyk, Lassila et al. 2012](#), [Amstrup, Sikjaer et al. 2015](#)). In the MOTS, decreases in the ratio of bone resorption to bone formation (i.e.,  $\downarrow$ CTx:P1NP) occur suggesting that MSDK may be renormalizing bone marker turnover towards equilibrium resulting in an increase in BMD and, if taken for extended periods of time, may continue to reverse bone loss through the aging process. These data also suggest that MSDK may be superior to melatonin alone since the findings from the MelaOst study suggest that the mechanism for melatonin-induced increases in BMD may be through calcium-mediated bone mineralization, which may produce a ceiling effect of melatonin alone on improving bone health if taken for long periods of time ([Amstrup, Sikjaer et al. 2015](#)).

In addition to this, the major increase in serum P1NP levels occurred during the first six months of treatment in the MOTS (graph shown in **Appendix VI**) which may indicate that MSDK is initiating bone formation in the first six months of treatment resulting in increases in BMD; and once bone mass is achieved to homeostatic levels, MSDK then begins to regulate bone remodeling to maintain equilibrium. This is important because too much bone growth may lead to osteopetrosis or an overgrowth of bone. MSDK-mediated increases in bone density and P1NP along with decreases in CTx indicate a dual anabolic and possibly antiresorptive effect of MSDK, making it unique with respect to current osteoporosis therapies.

Currently, no data exist with respect to therapeutic levels of melatonin on bone. Because of this, nocturnal urinary melatonin levels were assessed in both groups. Women taking MSDK (which contained 5mg melatonin) at night had ~140 times more nocturnal melatonin than women

taking placebo. In fact, women in the placebo group had very low endogenous nocturnal urinary melatonin-sulfate levels consistent with reports showing low melatonin levels in aged and menopausal population ([Bellipanni, Bianchi et al. 2001](#), [Witt-Enderby, Radio et al. 2006](#)). Even though women in the MSDK group took supplements for a year, their melatonin levels varied widely possibly due to bioavailability differences between women similar to what is seen in males (range: 10%-56% in men; mean 33%) ([Di, Kadva et al. 1997](#)). In the MOTS, a direct relationship between melatonin levels and lumbar spine BMD was observed and is consistent with the findings of Amstrup *et al.* that demonstrates a dose-dependent effect of melatonin on femoral neck BMD ([Amstrup, Sikjaer et al. 2015](#)). Therefore, exogenous melatonin supplementation alone or in combination with other bone-tropic agents like SDK may play an important role in maintaining bone density in postmenopausal osteopenic women.

Even though all MOTS participants could take up to 1000 IU of vitamin D<sub>3</sub> daily, serum vitamin D<sub>3</sub> levels were assessed to ascertain if the MSDK-mediated increases in BMD and P1NP were due to differences in vitamin D<sub>3</sub> levels between the groups. Surprisingly, serum vitamin D<sub>3</sub> levels in women taking MSDK did not differ significantly when compared to placebo, despite the fact that women taking MSDK would have a higher intake of vitamin D<sub>3</sub> over the course of one year. In fact, many of our MOTS participants still remained below the sufficient level (30-100ng/mL) according to the Endocrine Society Clinical Practice Guidelines ([Holick, Binkley et al. 2011](#)). This, perhaps, is due to variable bioavailability in women with respect to vitamin D<sub>3</sub>. Because it has been shown that low vitamin D<sub>3</sub> status is associated with high serum CTx levels ([Napoli, Strollo et al. 2014](#)), we wanted to determine if this same negative correlation occurred in our study. Consistent with ([Napoli, Strollo et al. 2014](#)), a negative correlation between vitamin D<sub>3</sub> and CTx was also observed in our cohort. Perhaps the wide variations in vitamin D<sub>3</sub> levels in our

study contributed, in part, to the lack of MSDK's effect on CTx levels.

C-reactive protein (CRP) levels were measured to assess if MSDK could lower CRP levels as another mechanism to explain its inducing effects on BMD. Levels of CRP are inversely correlated with vitamin D levels ([De Vita, Lauretani et al. 2014](#)) and BMD ([de Pablo, Cooper et al. 2012](#)). Human serum CRP (hsCRP) levels are significantly higher in lumbar spine osteoporotic women than in normal women ([Lee, Kim et al. 2011](#)); high circulating hsCRP levels (>1.8mg/L) are associated with postmenopausal osteopenia and/or osteoporosis ([Koh, Khang et al. 2005](#)); and fracture risk has been shown to occur in perimenopausal women who have CRP levels  $\geq 3$ mg/L ([Ishii, Cauley et al. 2013](#)). After one year of MSDK treatment, CRP levels of the MOTS participants dropped below 1.4mg/L with a mean group value of 0.57mg/L compared to the placebo mean value of 1.5mg/L. A reduction in CRP levels by MSDK could imply a possible anti-inflammatory role of MSDK, aiding in the bone health and other diseases such as cardiovascular and metabolic disorders.

Morphometric analyses were conducted to determine if any changes in body morphometry occurred in response to MSDK. No significant changes occurred in response to MSDK treatment when compared to placebo; however, there were two interesting observations regarding height and weight. Historical height loss greater than 6 cm is associated with the likelihood of vertebral fracture ([Siminoski, Warshawski et al. 2006](#)). In keeping with this, both MSDK and placebo groups experienced height loss (albeit at a much lower extent than 6cm) where women in the MSDK group lost 0.05% of their height while those in the placebo group had a height loss of 0.21%. Perhaps the gain in lumbar BMD in the MSDK group prevented loss of height and, if taken for longer periods of time, may lead to a decrease in vertebral fractures. Dramatic changes in weight could also contribute to increases in bone turnover and decreases in bone mass ([Labouesse,](#)

[Gertz et al. 2014](#)). Also, mature women with a BMI lower than 18kg/m<sup>2</sup> are estimated to have more than 30% bone loss than normal women of same age ([Emaus, Wilsgaard et al. 2014](#)). In our study, we observed no significant changes in mean weight within and between groups over the course of one year; however, there was more variance of weight change within the placebo group compared to the MSDK group ( $p= 0.032$ ). Perhaps MSDK, most likely through melatonin, stabilized weight fluctuation and provided some bone protection. Melatonin's effects on body weight has been reported in the MelaOst study where postmenopausal osteopenic women taking melatonin have a decrease in total fat mass and trended towards an increase in lean body mass ([Amstrup, Sikjaer et al. 2015](#)). Also, in Maria *et al* 2017, melatonin modulates the metabolic proteins, PPAR $\gamma$  and GLUT4, in a manner that would result in a lowering of fat production in the body (including bone marrow) while inducing osteoblast differentiation ([Maria, Samsonraj et al. 2017](#)). This shifting away from adipogenesis (i.e., decreased PPAR $\gamma$  and GLUT4 levels) towards osteogenesis may explain, in part, MSDK's actions on bone, which is supported in the co-culture studies (to be discussed in the next chapter) using human mesenchymal stem cells and peripheral blood monocytes and also in monocultures of mesenchymal stem cells derived from human adipocytes. Similar to melatonin, MSDK, in combination with osteogenic medium, significantly reduced PPAR $\gamma$  and GLUT4 expression consistent with MSDK's stimulatory effect on osteoblastogenesis and inhibitory effect on adipogenesis. These mechanisms and more are discussed later in the *in vitro* chapter.

Cardiovascular parameters, specifically blood pressure, was monitored during the recruitment process but also throughout the MOTS because the individual components of MSDK have been shown to produce differential effects on cardiovascular health. For example, strontium ranelate is associated with a risk of myocardial infarction (relative risk 1.6 vs. placebo) ([Bolland](#)

[and Grey 2013](#)). For the sake of comparison, this risk is lower than that of calcium (HR= 1.86 for dietary calcium and 2.39 for calcium only supplements ([Li, Kaaks et al. 2012](#)). Also, compared to bisphosphonates, strontium is not significantly associated with risk of acute coronary syndrome (rate per 1000 person-years 5.7 for strontium vs. 6.3 for alendronate/risedronate; adjusted HR 0.89, 95% CI 0.52 to 1.55) or any-cause mortality (adjusted HR 0.96, 95% CI 0.76 to 1.21) ([Svanström, Pasternak et al. 2014](#)). Melatonin and vitamin K<sub>2</sub> also have cardioprotective roles in the body ([Geleijnse, Vermeer et al. 2004](#), [Paradies, Paradies et al. 2015](#)). Besides, osteopenic patients tend to have less cardiovascular risk than osteoporotic patients, as observed in a study with Japanese postmenopausal women, where osteoporotic women have impaired endothelial function in their forearm resistance arteries compared to osteopenic women ([Sanada, Taguchi et al. 2004](#), [Farhat and Cauley 2008](#)). However, because of the lack of adverse reporting studies using strontium citrate, we could not exclude the possibility of having cardiovascular events with strontium citrate and therefore only recruited those women in the study who had normal blood pressure (BP). In addition to excluding women with elevated blood pressure level during the screening process, bimonthly BP assessments were performed in our study participants to detect any change in their BP while taking this therapy. No worsening effect of MSDK on blood pressure was observed throughout the study. Even though the baseline diastolic BP in the MSDK group was higher compared to placebo, their BP level remained steady and within the normal range throughout the study. MSDK did not affect systolic and diastolic BPs in our study throughout the treatment, making it potentially safe for use in the elderly population.

The second endpoint in this study was to assess MSDK's effects on the health-related QOL using validated questionnaires measuring menopausal symptoms, anxiety, stress and depression; and daily diaries. These parameters were measured because health related QOL becomes greatly

hampered in women transitioning through menopause. Studies showed that more than 60% postmenopausal women suffer from three or more menopausal symptoms, among which sleep disturbance, vaginal dryness and anxiety have the highest impact on their QOL. ([Greenblum, Rowe et al. 2013](#)). Unlike the MOPS ([Kotlarczyk, Lassila et al. 2012](#)), which shows an improved MENQOL physical domain score in perimenopausal women following 3 mg melatonin nightly for 6 months, a one-year MSDK supplementation did not significantly improve the physical symptoms associated with menopause and did not improve any of the other menopausal domains: psychosocial, vasomotor or sexual. These differences could be attributed to the fact that the study populations between the MOPS (perimenopausal) and the MOTS (postmenopausal) were quite disparate with respect to the menopausal transition. The prevalence of menopausal symptoms as well as physiologic distress associated with menopause is reportedly higher in perimenopause than postmenopause ([McKinlay, Brambilla et al. 1992](#), [Bromberger, Meyer et al. 2001](#)). This was also supported in our study where an improvement in participants' MENQOL psychosocial symptoms were significantly associated with an increase in their ages, indicating the women who are in postmenopausal state for a longer period compared to those who just entered postmenopause. This could possibly explain why we did not see significant QOL changes in these aspects in our postmenopausal cohort. Also, as discussed previously, vitamin D<sub>3</sub> supplementation may have prevented any vasomotor symptoms from surfacing since a negative correlation exists between vitamin D<sub>3</sub> and vasomotor symptoms. Though not significant, the sexual domain is the only domain that showed a splitting off into opposite directions for placebo (positive direction) and MSDK (negative direction); a more negative number indicates improvement. Items included in the sexual domain include: decrease in sexual desire, vaginal dryness, and avoiding intimacy. Participants in both groups maintained normal and/or healthy psychological states throughout the

study indicating that one-year MSDK treatment did not worsen their health-related QOL.

Daily diary information revealed additional treatment effects of MSDK on general well-being, which were not possible to capture from the specific questionnaires. A positive relationship between MSDK treatment and sleep quality occurred. This is an important finding considering that poor sleep quality, specifically going to bed at a later bedtime, sleeping late into the morning and frequent daytime napping is associated with low BMD in postmenopausal women ([Chen, Chen et al. 2014](#)). Also, disrupted sleep rhythms, as seen in shift workers, has also been shown to decrease BMD and increase one's risk of hip and wrist fracture ([Feskanich, Hankinson et al. 2009](#), [Quevedo and Zuniga 2010](#), [Kim, Choi et al. 2013](#), [Wang, Wu et al. 2015](#)). Another study shows an independent association between nighttime sleep problems with an increase in fall risk in an elderly population (aged 64-99 years) ([Brassington, King et al. 2000](#)). Therefore, an improvement in their sleep quality by MSDK may also contribute to the positive effects of MSDK on their bone health and fracture risk.

The melatonin component in MSDK is an efficacious agent for entraining sleep rhythms and this important fact may be improving bone health by regulating sleep quality and bone rhythms but also by improving compliance ([Maria and Witt-Enderby 2017](#)). Melatonin contained within MSDK may improve compliance due to its positive reinforcing effects on sleep quality and mood ([Maria, Samsonraj et al. 2017](#)). Because bone resorption (N-terminal peptide) follows a circadian rhythm that parallels melatonin's endogenous rhythm, as shown in premenopausal women ([St Hilaire, Rahman et al. 2018](#)), the preservation of the circadian rhythm by nocturnal exogenous melatonin supplementation may prevent the disruption of bone marker rhythms due to lifestyle (i.e., light at night, shift work, stress) and maintain balanced bone remodeling; this would keep one's sleep rhythms entrained to the light/dark cycle and produce benefits to bone and overall well-

being. This is supported in the MOTS and MelaOst trials where 29% more positive/neutral comments about sleep and 14% more positive comments about mood were made by women taking MSDK compared to placebo (MOTS) and a borderline significant improvement in sleep quality occurs after 1 year of melatonin treatment in a subgroup of postmenopausal osteopenic women with poor sleep quality ([Amstrup, Sikjaer et al. 2015](#)). Like melatonin, the strontium citrate component in MSDK may be contributing to improvements in QOL as well. In the SOTI trial, QOL was measured using the Quality of Life questionnaire in Osteoporosis (QualiOst), which is a validated disease-specific 23 items questionnaire measuring the effect of osteoporosis on the health-related QOL. Strontium ranelate treatment slightly improves the QOL in their postmenopausal osteoporotic cohort, which persisted for up to 4 years as reviewed ([Roux 2008](#)). Improvements in sleep duration cannot be factored into the improvement in QOL because both groups averaged ~7h of sleep per night throughout the study. This could be because almost all our participants were working women with a scheduled lifestyle and used alarm clocks to wake up in the morning.

Besides sleep, there were a high number of comments (both positive and negative) made about mood in both groups making it difficult to provide a satisfactory conclusion about MSDK's effect on general mood in this cohort. What is interesting to note, though, was that more positive comments were made in the MSDK group compared to placebo when neutral comments were excluded. Perhaps the improvement in sleep quality observed in the MSDK group translated to improvements in mood. In another study, melatonin shows general improvements in mood and depressive states in postmenopausal women ([Bellipanni, Bianchi et al. 2001](#)). Treatment effects on the GI tract were also evaluated because GI disorders, particularly nausea and diarrhea, are found to be associated with strontium ranelate usage in postmenopausal women ([Reginster,](#)



[Seeman et al. 2005](#)). In the MOTS, no effect of MSDK on GI symptoms occurred and, in fact, women taking MSDK, to some extent, demonstrated an improvement in GI-related matters. This was revealed by the number of positive comments made by women taking MSDK compared to all of the negative comments made by placebo group. A similar trend towards an improvement was observed in the general aches/pain category where MSDK produced minimal, if any, effects. These findings underscore the fact that MSDK is safe to use with respect to these aspects in postmenopausal women with osteopenia.

Treatment compliance has a great influence on the overall QOL. Bone loss therapies often fail to produce their desired effect because of the poor compliance and limited adherence to the treatments. One study shows that the compliance rate for taking osteoporosis medications is <80% as measured by the medication possession ratio (MPR) and this is associated with a 17% increase in fracture rate ([Silverman and Gold 2010](#)). Women taking MSDK were highly compliant throughout the study (92.4%) with no reported adverse events. Two of the participants dropped out from the study—one from placebo group due to having a problem with the pill size and another from the MSDK group due to general illness unrelated to the study supplement. Improved treatment compliance is expected to improve bone health outcomes in postmenopausal women with osteopenia.

## **Chapter 3: Assessment of mechanisms underlying the effect of melatonin, strontium citrate, vitamin D<sub>3</sub> and vitamin K<sub>2</sub> (MSDK) on human adult mesenchymal stem cells and human peripheral blood monocytes grown as co-cultures**

### **3.1. Materials and Methods**

#### **3.1.1. *In vitro* treatment preparation**

*In vitro* MSDK treatment concentrations were calculated based on the doses used in the MOTS clinical trial. Hence, 50 nM melatonin (M), 191.5 µM strontium citrate (S), 26 nM vitamin D<sub>3</sub> (D) and 18.5 nM vitamin K<sub>2</sub> (K) were prepared and dissolved into 100% pure ethanol to achieve the final concentration of MSDK per well. All study drugs were generously provided by Pure Encapsulations, Inc. (Sudbury, MA, USA).

#### **3.1.2. Osteoblast/Osteoclast co-cultures and hMSC mono-cultures**

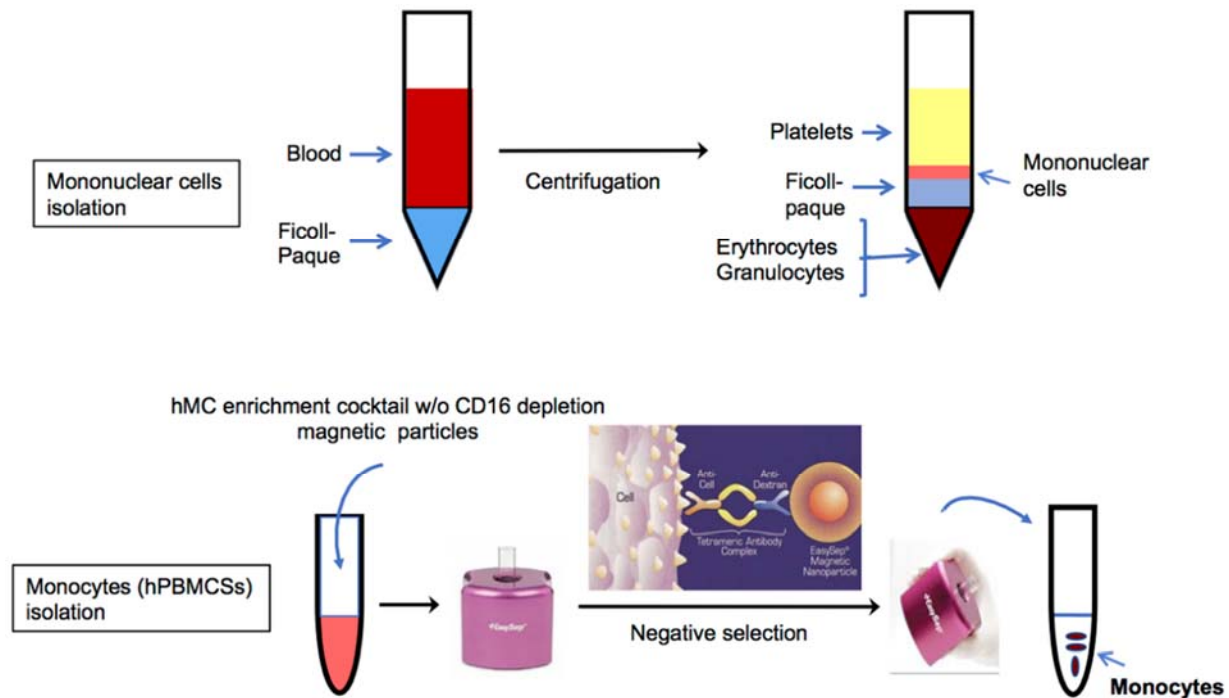
Two bone cell co-culture model systems (transwell and layered) were developed using undifferentiated forms of osteoblasts and osteoclasts—multipotent human adult mesenchymal stem cells were used to study osteoblastogenesis and human peripheral blood monocytes, isolated from freshly drawn human blood, were used to study osteoclastogenesis.

***Initiation of hMSCs culture.*** Human adult mesenchymal stem cells (hMSCs) (CAT# PT-2501, Lonza, MD, USA) were grown in 75 cm<sup>2</sup> cell culture flasks using mesenchymal stem basal cell growth medium (Os-) (CAT# PT-3001, Lonza, USA) and cells were maintained at 37°C, 5% CO<sub>2</sub> and 90% humidity. When 80% confluence was achieved, cells were passaged following detachment from the flask surface by trypsinization and transfer into other tissue culture plates or flasks. Cells were then seeded (at passage 3-5) at an initial density of  $3 \times 10^3$  cells/cm<sup>2</sup> at the bottom chamber of Corning<sup>TM</sup> transwell permeable support 6 well plates (Cat# 07-200-165; pore

size 0.4 $\mu$ m, 24.5mm Diameter, 4.7cm<sup>2</sup> Growth Area; Corning, USA) or on typical 6-well cell culture plates (Corning, NY, USA). On day 1 of the 21-day co-culture period, cells were treated with either basal growth media (Os-) or osteogenic media (Os+) (CAT# PT-3002, Lonza, USA) and then with 0.01% ethanol as vehicle (Veh) or melatonin (Mel), strontium citrate (Sr), vitamin D<sub>3</sub> (D3) and vitamin K<sub>2</sub> (K2) alone or in combination with MSDK. Ascorbate, dexamethasone and  $\beta$ -glycerophosphate were added to basal growth medium, referred to as osteogenic media (Os+), to induce the differentiation of hMSCs into osteoblasts ([Langenbach and Handschel 2013](#)). Full media exchanges occurred every four days.

***Isolation of hPBMCs from blood sample.*** On day 13, a blood sample (approx. 2mL) was taken from a young consenting healthy volunteer unrelated to the MOTS clinical trial via venous puncture using BD Vacutainer<sup>®</sup> Safety-Lok blood collection set with a 23-gauge needle (BD, USA) and collected in 8.5 mL BD Vacutainer<sup>®</sup> SST<sup>™</sup> Plus blood collection tubes with anticoagulant (BD, USA). To isolate peripheral mononuclear cells, anticoagulant-treated blood was mixed with an equal volume of balanced salt solution (e.g. PBS). The blood/PBS solution (1:1) was then carefully layered on top of the Ficoll-Paque<sup>™</sup> Plus solution (Amersham Pharmacia Biotech, Sweden) without disturbing that layer. After two repeated centrifugations at 400g for 30-40 min at 18-20<sup>0</sup>C without brake, mononuclear cells were visible as a yellowish layer in the middle. They were then isolated from the multiple layers of centrifuged blood using a long sterile pipette tip and re-suspended in Robosep<sup>™</sup> buffer (CAT# 20104, Stemcell technologies, USA). Magnetic separation of pure peripheral blood monocytes (hPBMCs) from the fresh mononuclear cells mixture was performed using the EasySep<sup>™</sup> negative selection human monocyte enrichment kit without CD16 depletion (CAT# 19058, Stemcell technologies, USA) and purple EasySep<sup>™</sup> magnet (CAT# 18000, Stemcell technologies, USA), following manufacturer's instructions.

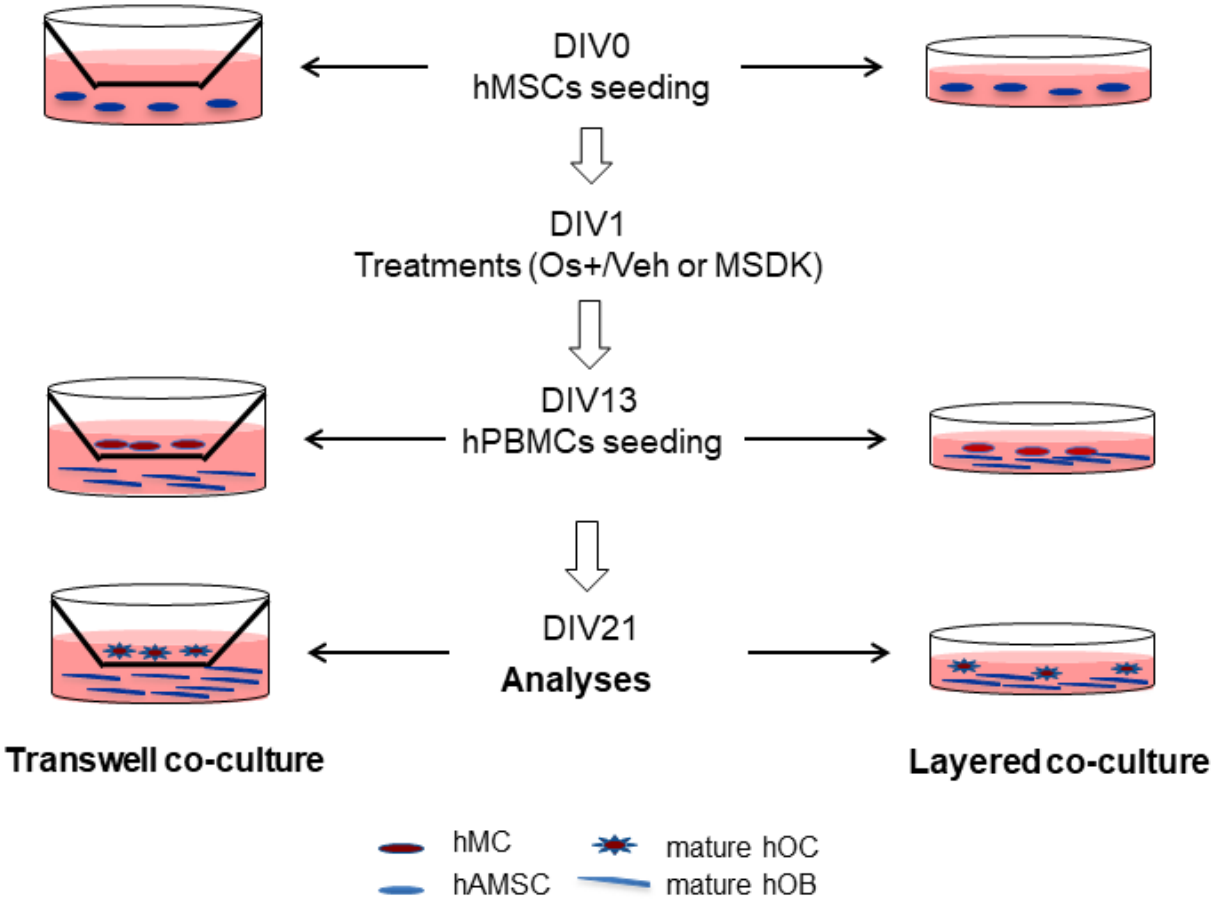
Briefly, mononuclear cell suspensions ( $\sim 5 \times 10^7$  cells/mL) in 2mL RoboSep buffer media were placed in a 5mL polystyrene tube (CAT# 352058, BD Bioscience, USA). Next, the tube was inserted into a magnet and the suspension was treated with EasySep™ human monocytes enrichment antibody cocktail without CD16 Depletion (50uL/mL cells) and incubated at 2-8°C for 10 min. Following antibody treatment, the mixture was treated with the magnetic particles (50uL/mL cells) and incubated for another 5 min at 2-8°C. The antibody cocktail provided with the kit bound to all mononuclear cells except CD16-specific monocytes. This complex also recognized dextran-coated magnetic particles provided with the kit. When the tube was placed inside the EasySep™ magnet and set aside for 2.5 min at room temperature, the magnetically labeled unwanted mononuclear cells remained bound to the inside wall of the tube, leaving only pure monocytes into the solution. Human peripheral blood monocytes (hPBMCs) in solution were then carefully poured into another tube. To avoid contamination of other cells, the tubes were not allowed to shake and the top of two tubes were not to touch each other. A schematic representation of this procedure is shown in **Figure 23**.



**Figure 23. Isolation of monocytes (hPBMCs) from blood sample**

**Initiation of hMSCs/hPBMCs co-culture.** Human PBMCs were added to osteoblastic cultures on day 13 because past studies using osteogenic medium have shown that hMSCs start to differentiate into mature osteoblasts between 14 to 21 days ([Sethi, Radio et al. 2010](#)) and begin producing substantial amounts of RANKL, M-CSF and/or OPG ([Atkins, Kostakis et al. 2003](#)) to modulate osteoclastogenesis ([Atkins, Kostakis et al. 2003](#), [Boyce and Xing 2008](#)). Monocytes were seeded ( $5 \times 10^3$  cells/cm<sup>2</sup>) in the top chamber of the transwell plate to initiate the “transwell” co-culture or layered directly on top of the hMSC culture to initiate “layered” co-culture. The permeable polycarbonate membrane present between the two chambers of the transwell allowed for the MSCs/osteoblasts and PBMCs/osteoclasts to communicate via passage of factors released into the media and not through contact whereas in the layered co-culture, MSCs/osteoblasts and PBMCs/osteoclasts could communicate via both means. Full media exchange was continued for once every four days until day 21.

**Initiation of hMSCs mono-culture.** The hMSC mono-cultures were cultured exactly as the co-cultures except that the hMSCs were grown in the absence of hPBMCs to determine how hPBMCs/osteoclasts influence MSDK-mediated osteoblast differentiation. The development and treatment paradigm of co-culture is shown in **Figure 24**.



**Figure 24.** Development of hMSCs/hPBMCs transwell (indirect) and layered (direct) co-cultures. DIV=Day *in vitro*.

### **3.1.3. Osteoblast differentiation and mineralization**

On day 21, calcium mineralization by matured, differentiated osteoblasts was measured via alizarin red staining assay— this time period was chosen based on past published studies using melatonin to induce differentiation of hMSCs into osteoblasts ([Sethi, Radio et al. 2010](#)). Alizarin red staining was performed on the bottom chamber (hMSCs portion) of transwell co-culture and directly on 6-well plates (both hMSCs and hPBMCs) of the layered co-culture using the commercially available osteogenesis quantification kit (CAT# ECM815, EMD Millipore, MA, USA) as per manufacturer's instructions. Briefly, the bottom chamber cells were washed with PBS and fixed with 10% formaldehyde and 15 min incubation at room temperature. Then alizarin red stain was applied to each well (0.5-1mL/well). Following 20 min incubation at room temperature and extensive washing (three times, 5 min, gentle rocking) with deionized water, differentiated osteoblasts containing mineral deposits were visualized using Vistavision microscope (VWR international) with progress C3 camera (Zenoptik).

Osteogenesis was also quantified by extracting the mineral deposits released by osteoblasts using the Osteogenesis Assay Kit (CAT# ECM815, EMD Millipore, MA, USA). Spectrophotometric quantification was performed at 405 nm using the Perkin Elmer Victor 1420 Multilabel plate reader (Waltham, MA, USA). A standard curve was generated from the absorbance (OD) readings of standards. Concentrations of alizarin red of the samples were calculated from the generated standard curve using Workout 2.0 software (Waltham, MA, USA), normalized against Os-/Veh and compared between groups. Osteoblastic mineralization activity was proportional to the concentration of alizarin red in this assay.

### **3.1.4. Osteoclast differentiation and resorption pit formation**

On day 21, tartrate resistant acid phosphatase (TRAP) assays were carried out on the top

chamber of transwell co-culture or directly on the 6-well plate (layered co-culture) to measure osteoclastic differentiation and TRAP releasing activity. Qualitative analysis was performed using the commercially available Acid Phosphatase Leukocyte assay kit (CAT# 387A, Sigma, USA) per manufacturer's instructions. Briefly, recommended reagents supplied in the assay kit were added to the cells (1mL/well) per kit instructions and incubated for 1h in a water bath at 37°C in the absence of light. The cells were then counterstained with hematoxylin for 2 min and rinsed thoroughly in alkaline tap water, resulting in the visualization of blue nuclei of osteoclasts. Microscopic assessment of the stained osteoclasts was performed using Vistavision microscope (VWR international) with progress C3 camera (Zenoptik) under grey setting. Purple staining indicated TRAP deposition by osteoclasts where the amount of TRAP deposition was proportional to the differentiation of osteoclasts.

Quantitative analysis of the total TRAP was performed according to the protocol previously explained by Janckila *et al.*, with modifications ([Janckila, Takahashi et al. 2001](#)). Briefly, Naphthol-ASBI phosphate (N-ASBI-P) was used as a substrate for TRAP. TRAP buffer was prepared by dissolving N-ASBI-P (2.5mM) in a solution containing 1% ethylene glycol monomethyl ether (EGME), 2% NP40, Na-acetate (100mM) and Na-tartrate (50mM) with pH adjusted at 5.5-6.1. Cells were lysed with 50mM TRIS and treated with TRAP buffer (1mL/well). Cells were also treated with a blank solution containing 100 µL TRIS and 1 mL TRAP buffer only. Cells were then scraped and then placed in a 5mL tube along with the buffer and incubated at 37°C for 30 min. Reactions were stopped upon the addition of 2.5 mL 0.1 M NaOH containing 0.05% NP-40. Fluorescence readings were taken at 405nm excitation and 515nm emission wavelength using a Perkin Elmer Victor 1420 Multilabel plate reader. Data were normalized against Os-/Veh and compared between groups. All reagents were bought from Sigma, USA. Osteoclastic



resorption pit formation activity was also performed by manually counting the number of resorption pits formed by the differentiated osteoclasts in the layered co-culture using a Vistavision microscope.

### 3.1.5. Western blot

Western blotting was performed to measure protein expression of osteoprotegerin (OPG), receptor activator of nuclear factor kappa-B ligand (RANKL), extracellular signal-regulated protein kinases 1 and 2 (ERK1/MAPK3 and MAPK1/ERK2), extracellular signal-regulated protein kinase 5 (ERK5/MAPK7), runt-related transcription factor 2 (RUNX2), integrin  $\beta$ 1 (ITGB1), nuclear factor kappa B (NF $\kappa$ B), peroxisome proliferator-activated receptors gamma (PPAR $\gamma$ ), glucose transporter type 4 (GLUT4/SLC2A4) and beta subunit of insulin receptor (IR $\beta$ ); using the Odyssey® Western Blotting Kit IV RD (CAT# 926-31084, Licor bioscience, USA). Primary antibodies included rabbit anti-OPG/TNFRSF11B (ab73400, Abcam, USA), rabbit anti-RANKL/TNFSF11 (ab9957, Abcam, USA), rabbit anti-phospho ERK1/2 (9101, Cell Signaling, USA), rabbit anti-total ERK1/2 (9102, Cell Signaling, USA), rabbit anti-phospho ERK5 (3371, Cell Signaling, USA), rabbit anti-total ERK5 (3372, Cell Signaling, USA), rabbit anti-RUNX2 (sc10758, Santa Cruz Biotech, USA), rabbit anti-Integrin  $\beta$ 1 (sc8978, Santa Cruz Biotech, USA), rabbit anti-NF $\kappa$ B (sc298, Santa Cruz Biotech, USA), rabbit anti-PPAR $\gamma$  (sc7196, Santa Cruz Biotech, USA), rabbit anti-GLUT4 (sc7938, Santa Cruz Biotech, USA), rabbit anti-IR $\beta$  (sc711, Santa Cruz Biotech, USA) and mouse anti- $\beta$ -actin (926-42212, Licor, USA). Secondary antibodies against appropriate IgG included goat anti-rabbit (800nm) and goat anti-mouse (680nm), which were supplied with the Licor western blotting kit.

***Cell lysate preparation.*** On day 21, osteoblast and osteoclast cell lysates each were prepared from the bottom and top chambers of the transwell co-culture, respectively. Whole cell lysates

containing both osteoblasts and osteoclasts were prepared from the layered co-culture. Following aspiration of culture media, cell lysates were prepared by adding 2X Laemmli sample buffer (CAT# 161-0737, BioRad, USA) and  $\beta$ -mercaptoethanol (CAT# 161-0710, BioRad, USA) at a ratio of 19:1 onto each well and then gently scraping the cells. Cell lysates were then heated for 5 min at 95°C, cooled down and stored at -20°C until use.

**SDS-PAGE.** Thirty microliters of cell lysates and 10  $\mu$ L of the molecular weight marker (Precision Plus Protein™, CAT# 161-0373, BioRad, USA) were added to wells and then separated using 10% SDS-polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose membranes and placed in blocking buffer for 1h with gentle rocking to reduce non-specific staining. Membranes were then incubated with the respective primary antibodies and anti- $\beta$ -actin antibodies to visualize the proteins of interest and to normalize protein load overnight at 4°C with gentle rocking. Following incubation with the antibodies, blots were washed with PBS-tween and incubated with two different secondary antibodies with different infrared spectra (800nm and 680nm) for 45min to 1 hour at room temperature. Protein bands were then visualized in Odyssey Infrared Imager and quantified using Odyssey software (Licor bioscience, USA). Proteins were normalized against  $\beta$ -actin to control for variations in protein loading between treatment groups. Protein levels were then normalized against Os-/Veh or Os-/MSDK and compared between groups.

### **3.1.6. Measurement of secreted OPG and RANKL**

Concentrations of osteoblast-secreted osteoprotegerin (sOPG) and receptor activator of nuclear factor kappa-B ligand (sRANKL) in culture media were measured via Sandwich enzyme-linked immunosorbent assay (ELISA using Osteoprotegerin Human ELISA kit (CAT# ab100617, Abcam, USA) and total sRANKL (human) ELISA kit (CAT# ALX-850-019, Enzo Life Science, USA), respectively, following manufacturer's instructions. Culture media was collected before

preparing the cell lysates from the bottom chamber of the transwell plate (contains hMSCs) or from the layered or hMSC mono-cultures and stored at -20°C until use. Absorbance readings of standards, controls and samples were measured at 450 nm using the Perkin Elmer Victor 1420 Multilabel plate reader (Waltham, MA, USA). A standard curve was generated for each assay using the four-parameter logistic curve fit function and concentrations of sOPG and sRANKL were calculated using Workout 2.0 software (Waltham, MA, USA). Mean concentration changes of sOPG (in pg/mL) and sRANKL (in pg/mL) were calculated, normalized against Os-/MSDK groups and compared between groups. Ratios of sOPG to sRANKL were calculated, normalized against Os-/MSDK and then compared between groups.

### **3.1.7. Statistical interpretation**

For *in vitro* assays, all data were normalized against either Os-/Veh or Os-/MSDK and analyzed by one-way ANOVA followed by Bonferroni's post-hoc multiple comparison *t*-test, where significance was defined as  $p < 0.05$ .

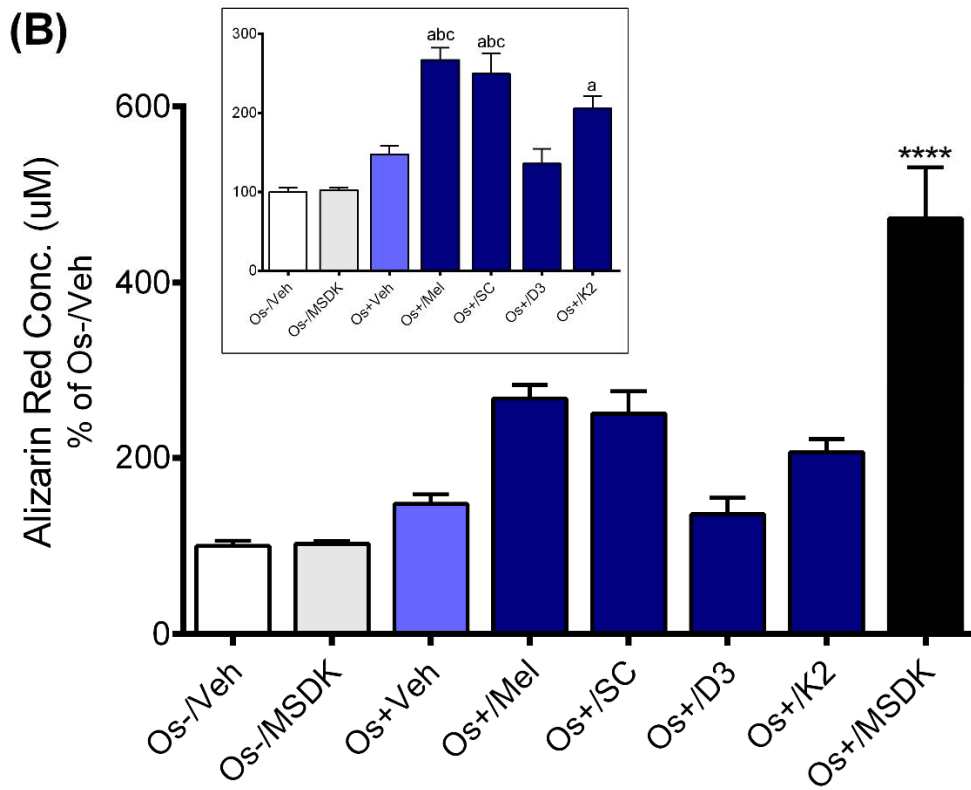
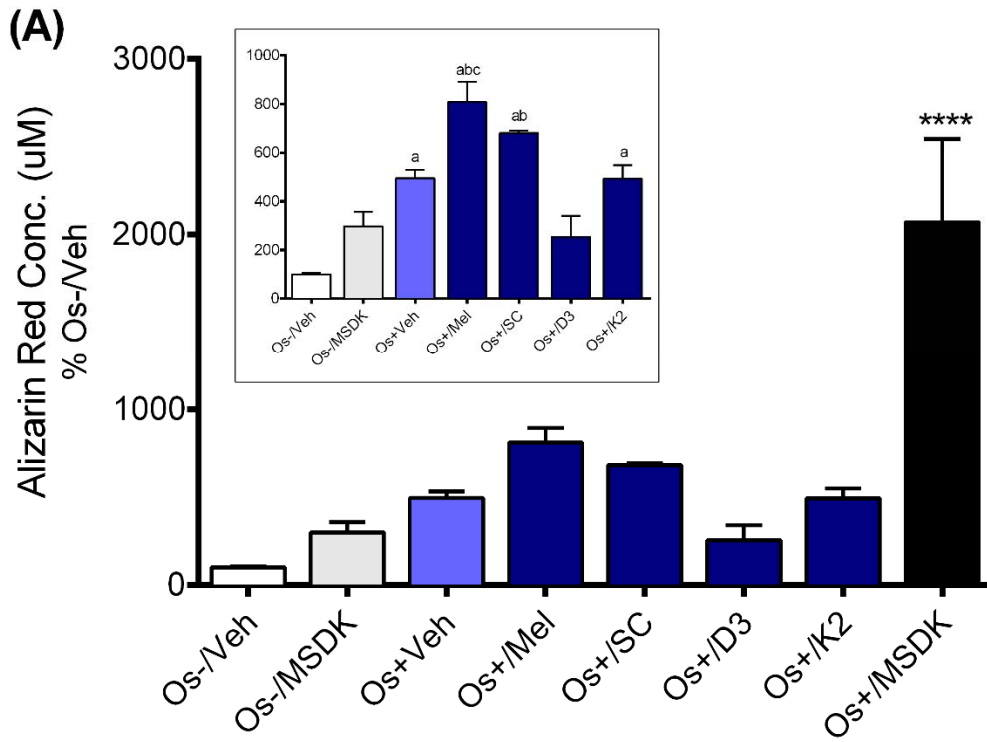
## **3.2. Results**

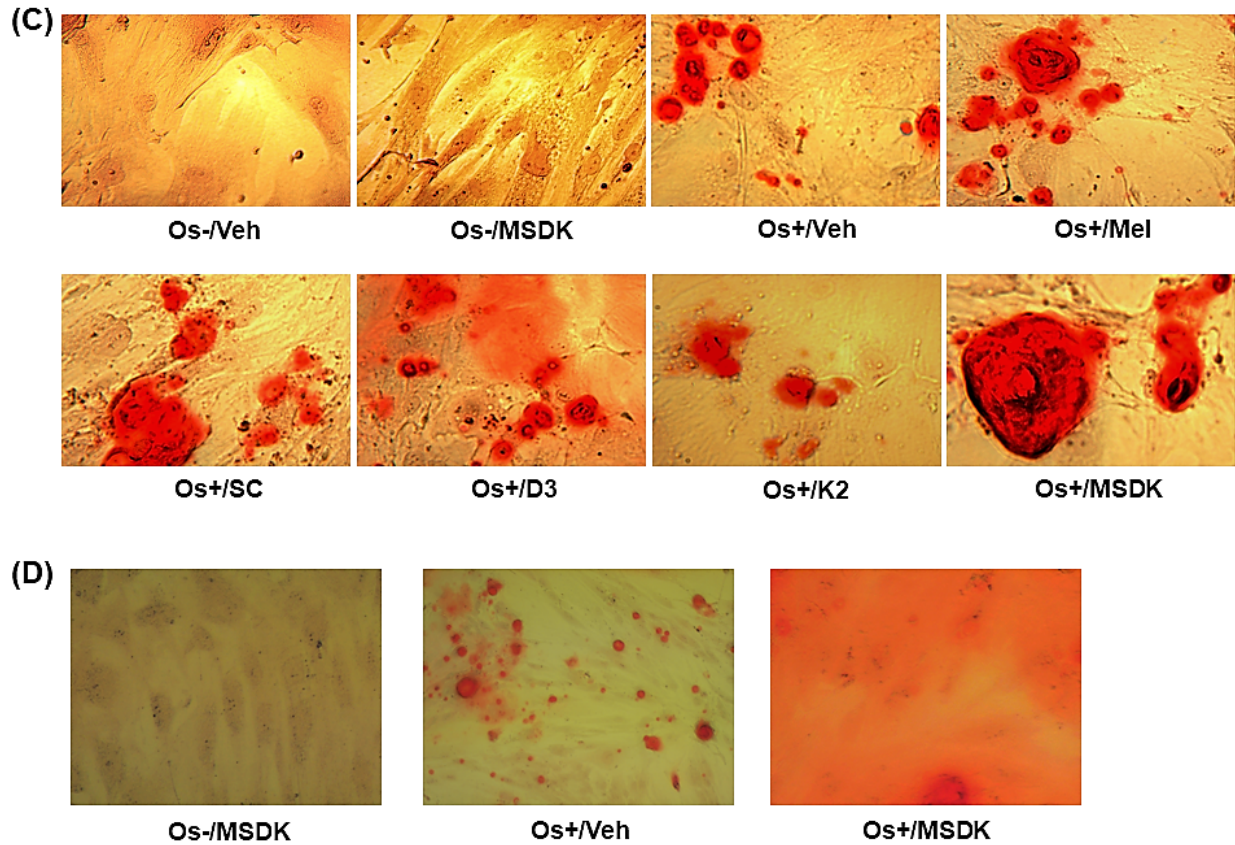
### **3.2.1. MSDK increased osteoblastogenesis and decreased osteoclastogenesis in co-cultures of hMSCs and hPBMCs**

Both alizarin red staining and TRAP assays indicated successful differentiation of hMSCs into osteoblasts and hPBMCs into osteoclasts when grown together as co-cultures in the presence of osteogenic (Os<sup>+</sup>) media. **Figure 25A** represents calcium mineralization activity of mature, differentiated osteoblasts grown in a transwell co-culture as measured after 21 days of exposure to MSDK and other treatments. Human adult MSCs grown in growth media alone (Os-/Veh) or in presence of MSDK (Os-/MSDK) did not differentiate into osteoblasts as revealed by the absence

of alizarin red staining. As expected, hMSCs exposed to osteogenic media alone (Os+/Veh) differentiated into osteoblasts ( $p < .01$  vs. Os-/Veh). The addition of MSDK to osteogenic media (Os+/MSDK) enhanced osteoblast mineralization to the greatest extent ( $p < .0001$  vs. all groups). We next investigated if the individual components of MSDK were capable of inducing osteoblast differentiation beyond that of Os+ alone. Therefore, melatonin (Mel), strontium citrate (SC), vitamin D<sub>3</sub> (D3) or vitamin K<sub>2</sub> (K2) each were added to Os+ media. Melatonin was the only component that increased transwell osteoblast differentiation vs. Os+/Veh (**Figure 24A** inset).

Similar treatment effects on osteoblast differentiation were observed in the layered co-culture except that the extent of MSDK-mediated osteoblast differentiation was less compared to the transwell osteoblasts (**Figure 25B**). However, both melatonin and strontium citrate induced osteoblast differentiation alone when compared to Os+/Veh in this co-culture system (**Figure 25B** inset). Qualitative alizarin red staining analyses showing the extent of calcium deposition further demonstrated the effects of MSDK and other treatments on transwell co-culture (**Figure 25C**) and layered co-culture (**Figure 25D**).

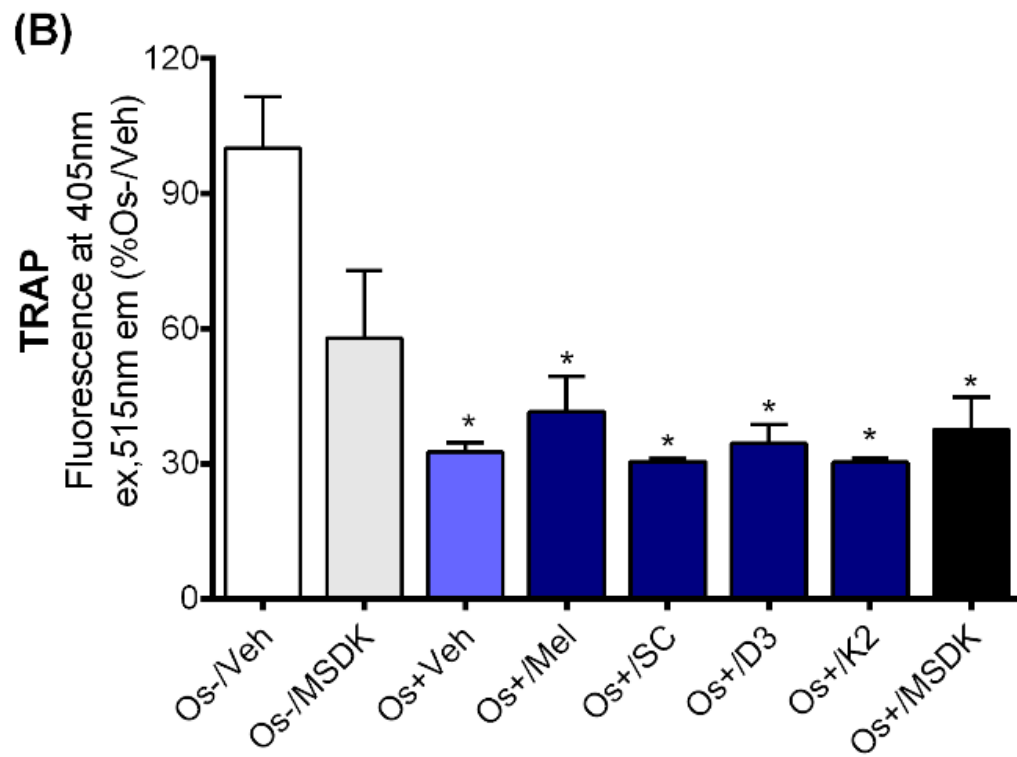
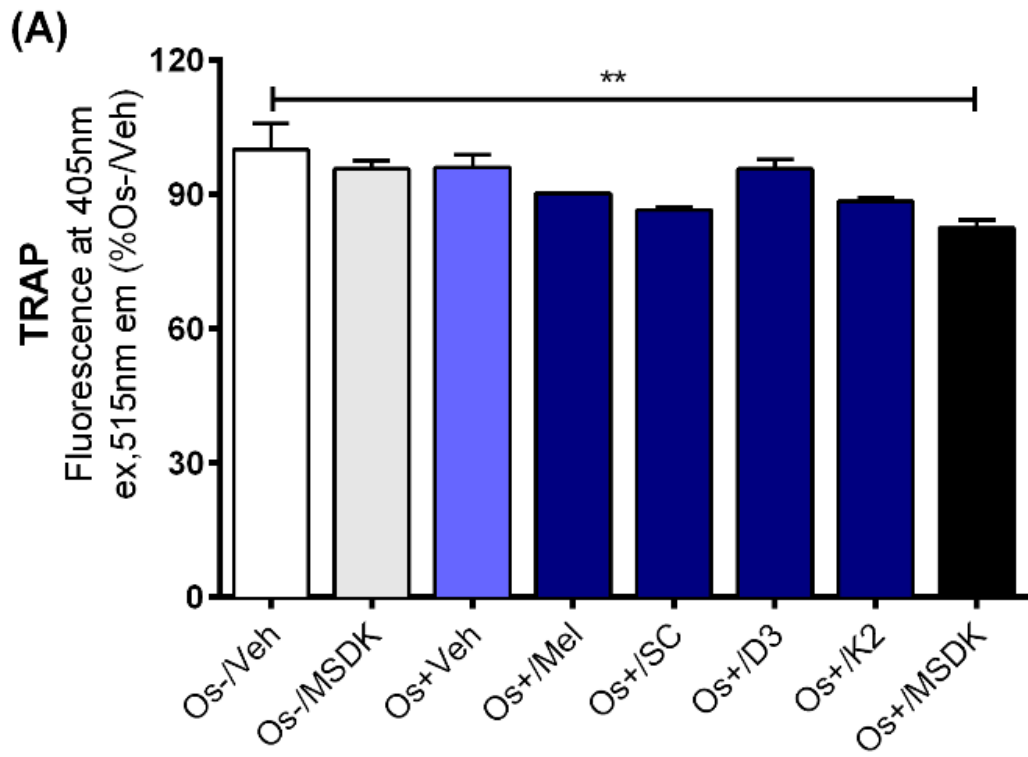




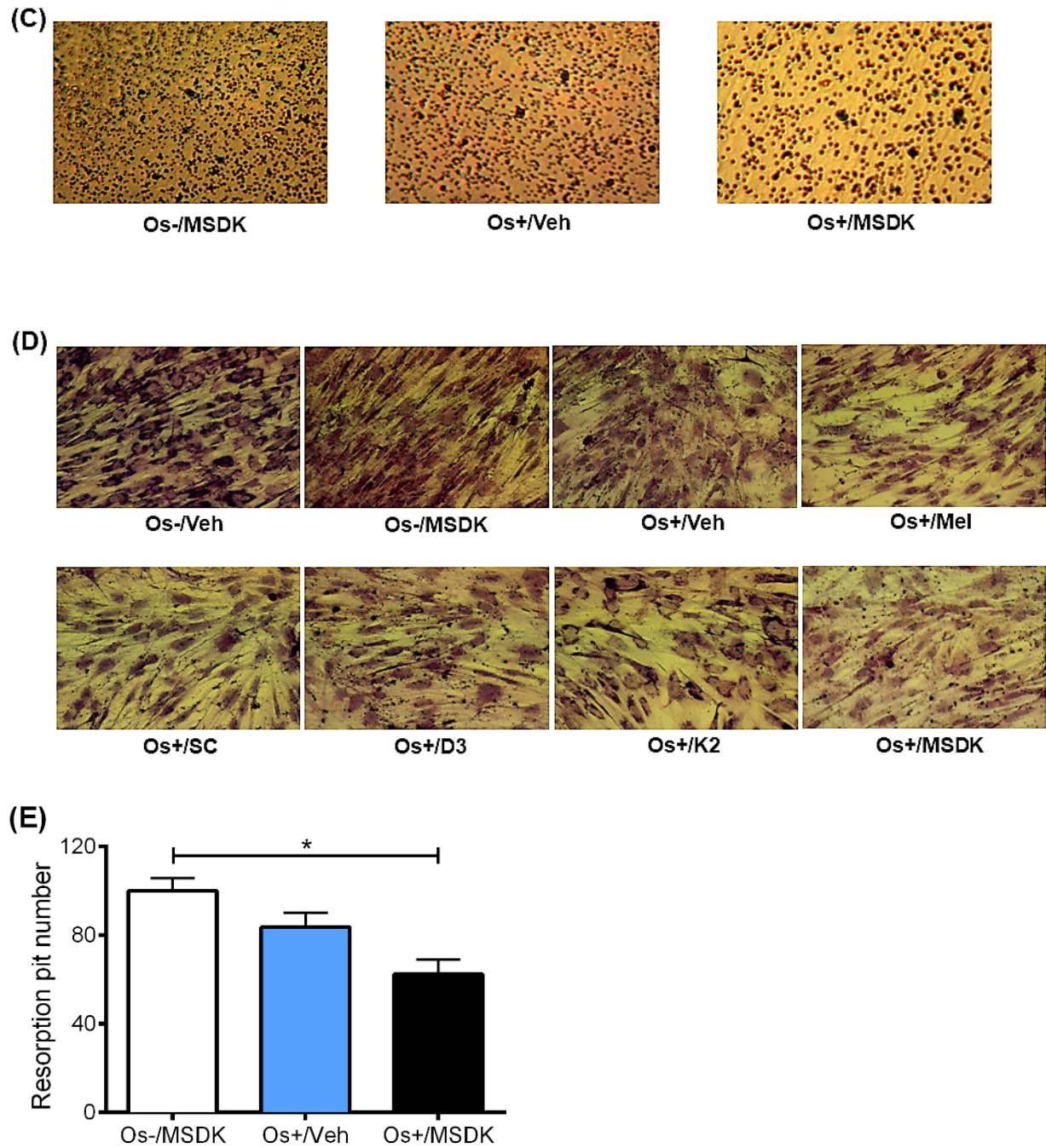
**Figure 25. Effect of MSDK on osteoblast-mediated calcium mineralization.** Following 21 days of exposure to MSDK and other treatments, calcium deposition by differentiated, matured osteoblasts was evaluated by alizarin red staining on **(A)** bottom chamber cells of the transwell co-culture or **(B)** in the layered co-culture. Each bar represents the mean ( $\pm$  S.E.M.) concentration of alizarin red ( $\mu$ M) for the respective group normalized against Os-/Veh. Inset graph represents similar analysis in the absence of Os+/MSDK. One-way ANOVA followed by Bonferroni's post-hoc multiple comparison *t*-test ( $n=3$  per group). Transwell co-culture: \*\*\*\*= $p<.0001$  vs. all groups,  $a=p<.01$  vs. Os-/Veh,  $b=p<.05$  vs. Os-/MSDK,  $c=p<.05$  vs. Os+/Veh. Layered co-culture: \*\*\*\*= $p<.0001$  vs. all groups,  $a=p<.01$  vs. Os-/Veh,  $b=p<.01$  vs. Os-/MSDK,  $c=p<.01$  vs. Os-/Veh). Representative images obtained from the qualitative assessment of osteoblast mineralization performed on the **(C)** bottom chamber cells of transwell co-culture and **(D)** layered

co-culture via qualitative alizarin red staining. Red color indicates calcium deposition by osteoblasts. Os<sup>-</sup> =basal media, Os<sup>+</sup> =osteogenic media, Veh= vehicle, Mel= melatonin, SC= strontium citrate, D3= vitamin D<sub>3</sub> (Cholecalciferol), K2= vitamin K<sub>2</sub> (MK7).

The effect of MSDK on TRAP levels was measured since TRAP is a well-known marker for terminally differentiated osteoclasts and their bone resorption activity ([Angel, Walsh et al. 2000](#), [Halleen, Tiitinen et al. 2006](#)). **Figure 26A** demonstrates treatment effects of MSDK and its individual components on TRAP releasing activity of the osteoclasts grown as transwell co-cultures. Although osteogenic media (Os<sup>+</sup>) favored transwell osteoblast formation, it did not have any effect on transwell osteoclast activity. However, the addition of MSDK to osteogenic media (Os<sup>+</sup>/MSDK) significantly inhibited TRAP expression, suggesting an inhibitory role of MSDK on osteoclast differentiation and activity. The individual components did not inhibit TRAP expression in this co-culture. **Figure 26B** illustrated treatment effects on TRAP expression in layered co-cultures. Os<sup>+</sup> was the only culture condition that inhibited TRAP expression. The addition of melatonin, strontium citrate, vitamin D<sub>3</sub> and vitamin K<sub>2</sub> to Os<sup>+</sup> media either alone or in combination as MSDK did not have any enhancing or inhibitory effects on TRAP expression. Qualitative TRAP expression by differentiated osteoclasts, as performed via Acid Phosphatase, Leukocyte assay (TRAP staining assay) in transwell and layered co-cultures are shown in **Figure 26C** and **D**, respectively. Qualitative illustrations matched to what was obtained from the quantitative analysis. Resorption pit number, as measured in the layered co-culture, significantly decreased in Os<sup>+</sup> culture treated with MSDK (**Figure 26E**), indicating an inhibitory effect of MSDK on osteoclastic pit forming activity.







**Figure 26. Effect of MSDK on osteoclast differentiation and resorption pit formation.** Following 21 days of exposure to MSDK and other treatments, TRAP releasing activity by differentiated, mature osteoclasts were evaluated by quantitative TRAP assays on (A) the top chamber of cells from the transwell co-cultures or (B) in the layered co-cultures. Each bar represents the mean ( $\pm$

S.E.M.) fluorescence reading of TRAP (at 405nm ex, 515nm em) for the respective group normalized against Os-/Veh. One-way ANOVA followed by Bonferroni's post-hoc multiple comparison *t*-test (n=3 per group). Transwell co-culture: \*\*= $p < .01$  vs. Os-/Veh. Layered co-culture: \*= $p < .05$  vs. Os-/Veh). Representative images obtained from the qualitative assessment of osteoclast differentiation performed on the **(C)** top chamber cells of transwell co-culture and **(D)** layered co-culture via qualitative Acid Phosphatase, Leukocyte assay (TRAP staining assay). The purple color indicates TRAP deposition by matured osteoclast which was further observed by blue nuclei of osteoclasts. Os- =basal media, Os+ =osteogenic media, Veh= vehicle, Mel= melatonin, SC= strontium citrate, D3= vitamin D<sub>3</sub> (Cholecalciferol), K2= vitamin K<sub>2</sub> (MK7). **(E)** Resorption pit number counted in layered co-cultures as a measure of osteoclast-mediated resorption pit formation activity. Each bar represents the number of resorption pit in the respective group. One-way ANOVA followed by Bonferroni's post-hoc multiple comparison *t*-test (n=3; \*= $p < .05$  vs. Os-/MSDK).

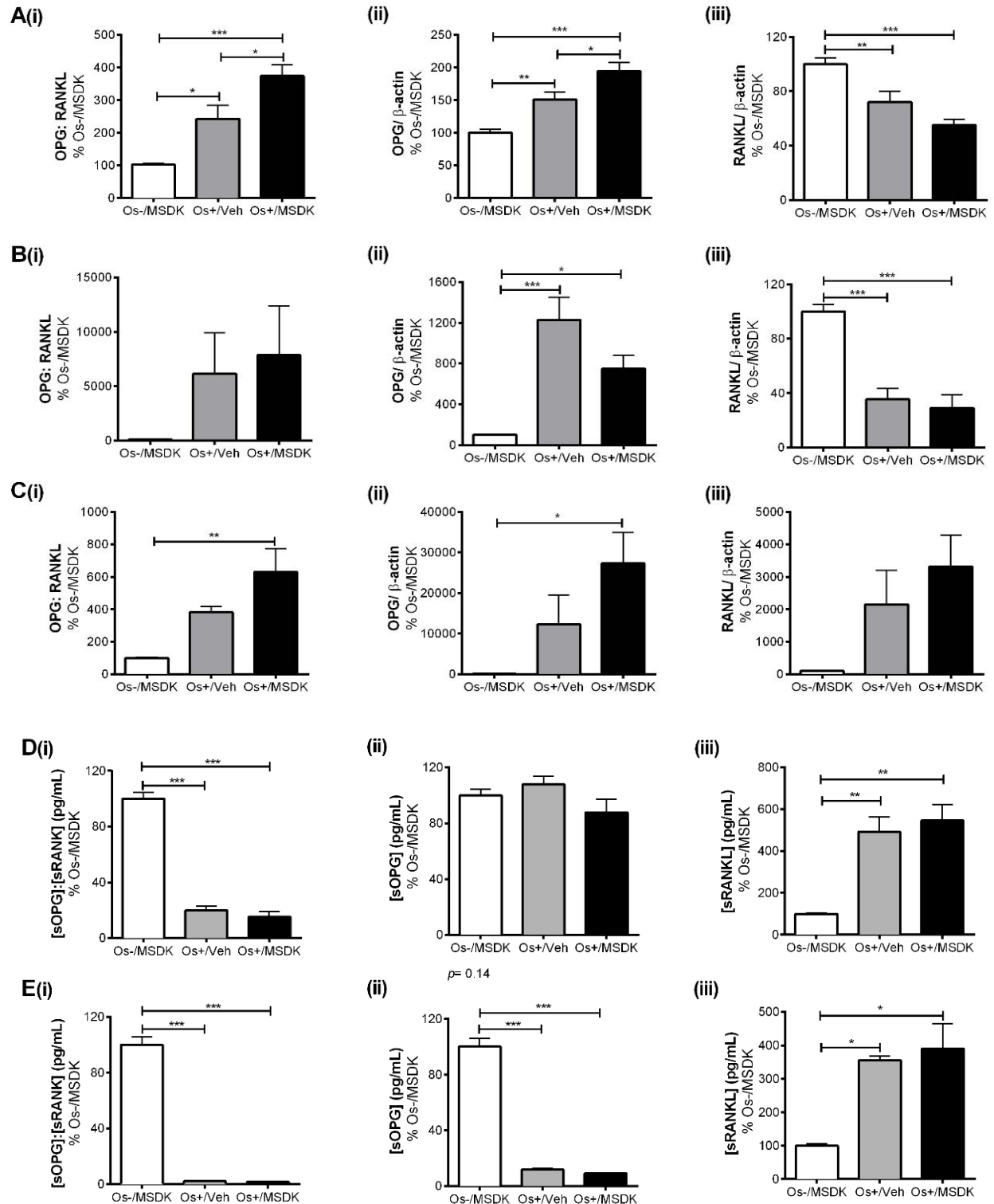
In summary, 21 days of MSDK treatment significantly enhanced osteoblast differentiation and mineralization in both transwell and layered co-cultures. MSDK treatment showed a parallel decrease in osteoclast differentiation and TRAP releasing activity in the transwell co-cultures. No additional inhibitory effect of MSDK on osteoclast differentiation was observed in layered co-cultures because Os+ alone produced substantial inhibition. Nonetheless, the manner in which osteoblasts and osteoclasts are cultured influences mostly the magnitude of osteoblast or osteoclast differentiation.

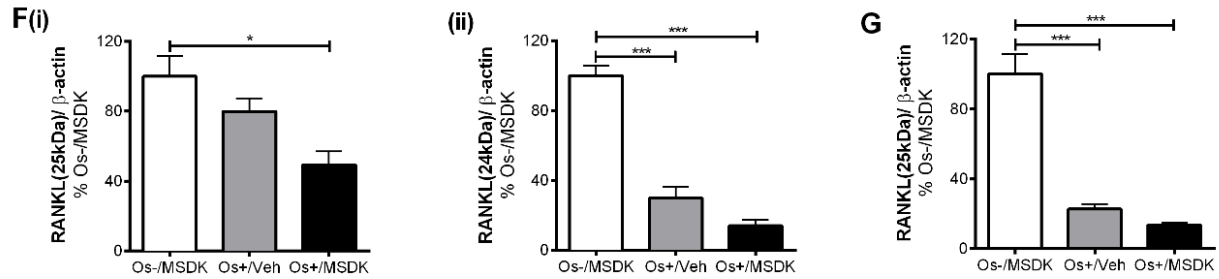
### 3.2.2. MSDK modulates OPG and RANKL levels in co-cultures of hMSCs and hPBMCs dependent upon the type of culturing condition—layered or transwell

**Figure 27** illustrates the effect of MSDK on osteoprotegerin (OPG) and receptor activator of nuclear factor kappa-B ligand (RANKL)—signaling proteins known to modulate osteoclast activity and differentiation. As shown in **Figure 27Ai**, Os<sup>+</sup> media alone significantly increased OPG and decreased RANKL expression in transwell osteoblasts, which also resulted in an increase in the OPG: RANKL ratio, as compared to growth media (Os<sup>-</sup>). The presence of MSDK in Os<sup>+</sup> media (Os<sup>+</sup>/MSDK) further enhanced the ratio of OPG: RANKL ( $p < .001$  vs. Os<sup>-</sup>/MSDK and  $p < .05$  vs. Os<sup>+</sup>/Veh), by increasing OPG (**Figure 27Aii**) and concomitantly decreasing RANKL (**Figure 27Aiii**) expression of transwell osteoblasts. Similar effects were observed when hMSCs were grown as mono-cultures (**Figure 27Ci** and **Cii**) in the absence of hPBMCs except that RANKL remained unchanged (**Figure 27Ciii**) at levels similar to control. Interestingly, when hMSCs were cultured in direct contact with hPBMCs (layered co-culture), no further enhancement of OPG (**Figure 27Bii**) or decrease in RANKL (**Figure 27Biii**) occurred with MSDK treatment vs. Os<sup>+</sup>/Veh, resulting in no MSDK-mediated increases in the ratio of OPG:RANKL (**Figure 27Bi**).

**Figure 27D** illustrates the effect of MSDK on OPG and RANKL secreted from transwell osteoblasts into the culture media, as measured via ELISA. The ratio of sOPG: sRANKL decreased in both Os<sup>+</sup>/Veh and Os<sup>+</sup>/MSDK treated co-cultures as compared to Os<sup>-</sup>/MSDK mostly due to significant increases in sRANKL. Similar patterns in sOPG: sRANKL levels were also observed in hMSC mono-cultures (**Figure 27E**) due to increases in sRANKL levels and decreases in sOPG levels. High levels of sRANKL (**Figure 27Diii** and **Eiii**) was expected to increase osteoclastogenesis, but this did not occur as shown in **Figure 26**. In fact, osteoclastogenesis was

inhibited in both co-cultures in the presence of osteogenic (Os+) medium and, even more so, in transwell co-cultures containing MSDK. The fact that OPG was the only protein modulated by MSDK and that its level correlated with osteoclastogenesis suggests that sOPG and not sRANKL was modulating osteoclastogenesis. Therefore, the attenuation of osteoclastogenesis in transwell co-cultures vs. layered may be due to the secretory pattern of OPG (sOPG) from the osteoblast rather than sRANKL (**Figure 27D and E**). Moreover, the decreased sOPG level in hMSC mono-cultures and the unchanged sOPG level in transwell co-cultures in response to MSDK suggest that the presence of osteoclast in the transwell co-culture is probably modulating sOPG release from the osteoblast. The inhibition of osteoclastogenesis observed in transwell co-cultures was possibly due to OPG-mediated decreases in free RANKL; this would decrease RANK activation on osteoclasts resulting in the decreases in osteoclastogenesis and activity. Another possibility is that differential processing of RANKL by proteinases located on the osteoblasts created soluble RANKL products, which were less capable of generating osteoclasts ([Nakashima, Kobayashi et al. 2000](#)). This was supported in **Figure 27F and G**, which demonstrated that MSDK uniquely modulated the expression of different RANKL peptide fragments in transwell and layered osteoblasts. The RANKL fragments, 25kDa (**Figure 27Fi and G**) and 24kDa fragments (**Figure 27Fii**), also referred to as “shredded fragments” indicate ectodomain shedding by A disintegrin and metalloproteinase domain-containing protein (ADAM) 10 or Matrix metalloproteinase (MMP) 14 ([Hikita, Yana et al. 2006](#)).





**Figure 27. Effect of MSDK on OPG and RANKL expression of osteoblasts in transwell co-cultures, layered co-cultures and hMSCs mono-cultures.** Following 21 days of MSDK exposure, (A-C) treatment effects on the OPG and RANKL expression of osteoblasts was measured via western blot in (A) transwell co-cultures, (B) layered co-cultures and (C) hMSC mono-cultures. Cell lysates were prepared on day 21 from the bottom (osteoblasts) chamber in the transwell co-culture or from the whole plate (both osteoblasts and osteoclasts) in the layered co-culture. Protein levels were normalized against  $\beta$ -actin and then to Os-/MSDK. Each bar represents the mean ( $\pm$  S.E.M.) expression of (i) OPG: RANKL, (ii) OPG and (iii) RANKL for respective cultures. (D, E) Treatment effects on osteoblast-mediated secretion of OPG (sOPG) and RANKL (sRANKL) were measured by ELISA in (D) transwell osteoblasts and (E) hMSC mono-cultures. Following 21 days of MSDK exposure, protein concentrations (in pg/mL) were analyzed in culture media and then normalized against Os-/MSDK. Each bar represents the mean ( $\pm$  S.E.M.) concentrations of (i) sOPG: sRANKL, (ii) sOPG and (iii) sRANKL for respective cultures. (F, G) Treatment effects on the extracellular portion of RANKL in osteoblasts were detected via western blot by measuring mean osteoblastic expression of the (i) 25 KDa and (ii) 24 KDa RANKL fragments in (F) transwell co-cultures; and (G) the 24 KDa RANKL fragment in layered co-cultures.  $*=p < .05$ ,  $**=p < .01$  and  $***=p < .001$ ; One-way ANOVA followed by Bonferroni's post-hoc multiple comparison *t*-test (n=6 per group).

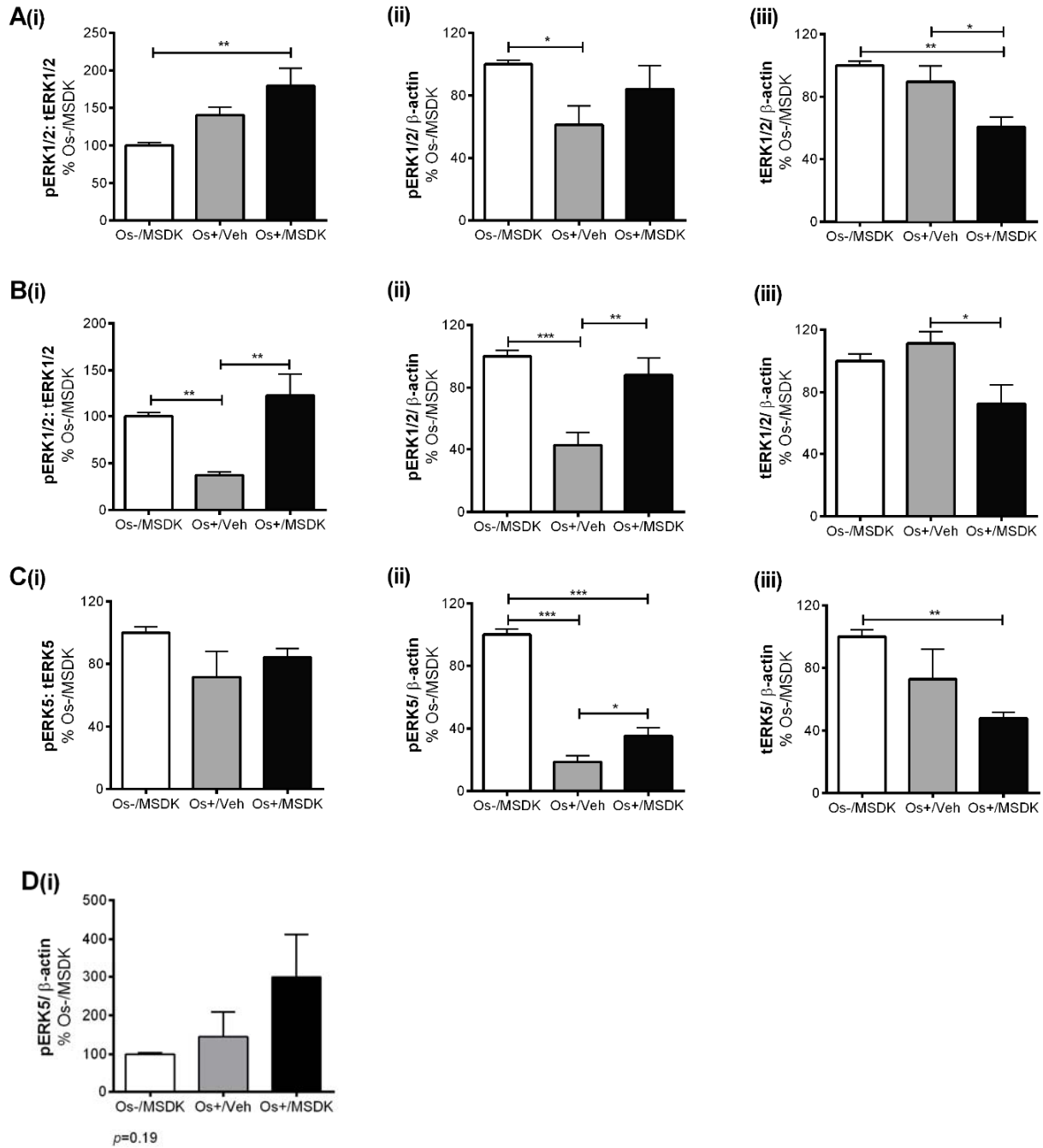
### 3.2.3. MSDK modulates pERK1/2 and pERK5 levels in co-cultures of hMSCs and hPBMCs dependent upon the type of culturing condition—layered or transwell

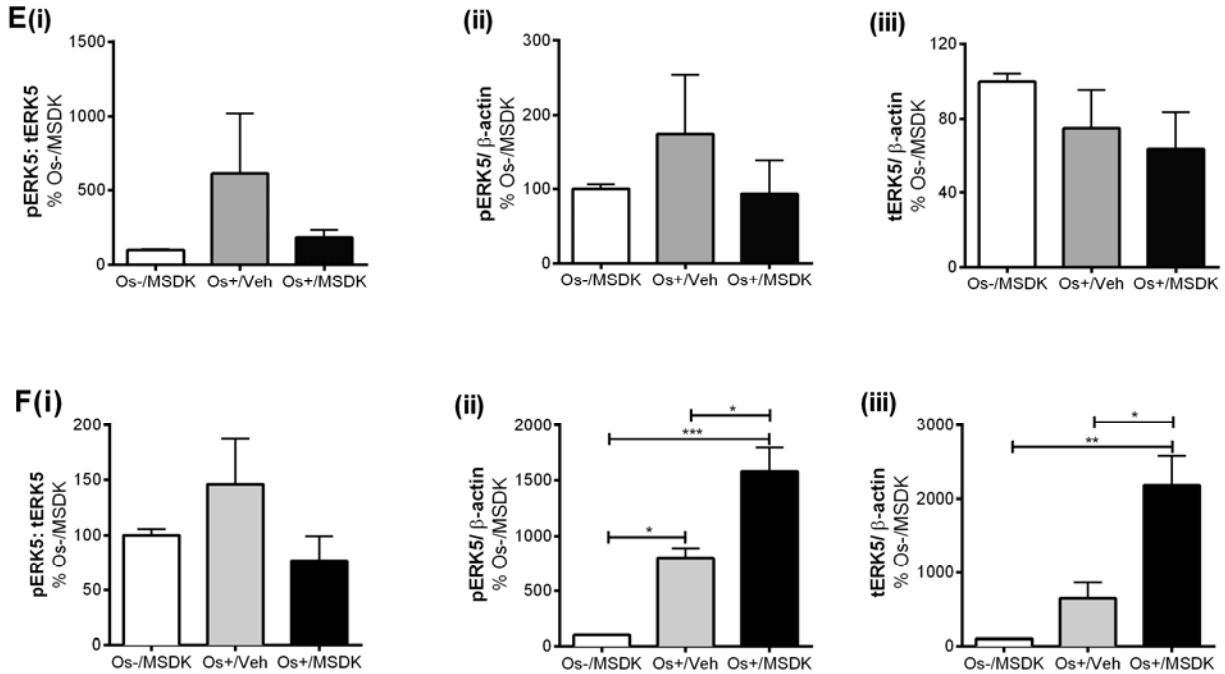
Studies done in our lab and others ([Radio, Doctor et al. 2006](#), [Ge, Xiao et al. 2007](#), [Ge, Xiao et al. 2009](#), [Matsushita, Chan et al. 2009](#), [Maria, Samsonraj et al. 2017](#)), reveal that melatonin induces osteoblast differentiation through MEK1/2. In hMSC mono-cultures, melatonin enhances their differentiation into osteoblasts by activating the ERK1/2 pathway because inhibition of MEK1/2 by PD98059 during the melatonin exposure period blocks these effects ([Radio, Doctor et al. 2006](#), [Sethi, Radio et al. 2010](#)). Since melatonin is one of the four components of MSDK, we examined whether ERK1/2 was regulated by MSDK in a manner like that of melatonin to make an association between ERK1/2 activity and expression to MSDK-mediated increases in osteoblastogenesis. **Figure 28A** represents the effect of MSDK on ERK1/2 activity and expression in osteoblasts grown as transwell co-cultures. The presence of MSDK in the Os<sup>+</sup> medium primarily enhanced ERK1/2 activity (phospho ERK1/2) and down-regulated total ERK1/2 (tERK1/2) in osteoblasts. This is consistent with previous data showing that ERK1/2 is associated with an increase in cellular differentiation ([Ge, Xiao et al. 2007](#), [Ge, Xiao et al. 2009](#), [Matsushita, Chan et al. 2009](#)), or, in this case, mesenchymal stem cell differentiation into osteoblasts. As shown in **Figure 28B**, MSDK produced a similar increase in ERK1/2 activity by both increasing phospho-ERK1/2 and decreasing total ERK1/2 expression which could be due to increases in phospho-ERK1/2 activity in osteoblasts, osteoclasts or both. In another study, ERK1/2 is also expressed in osteoclasts and plays a significant role in osteoclast differentiation ([Matsushita, Chan et al. 2009](#)). Therefore, it was not possible to conclude that MSDK was mediating ERK1/2 expression solely in osteoblasts in this co-culture. This could explain the difference in ERK1/2 expression between co-cultures with respect to MSDK.

Previous studies have shown that ERK5 also plays a role in both osteoblast and osteoclast function and, possibly, differentiation ([Li, Ma et al. 2012](#), [Kaneshiro, Otsuki et al. 2015](#), [Bo, Bin et al. 2016](#)). However, a recent study has shown that PD98059 and U0126, purported selective inhibitors of MEK1/2, also can inhibit MEK5 ([Drew, Burow et al. 2012](#)). These findings suggest that use of PD98059 or U0126 to assess the role of ERK1/2 in osteoblast proliferation and differentiation may have inadvertently inhibited both ERK1/2 and ERK5. Therefore, MSDK effects on ERK5 in all three-cell culture models—transwell, layered and monolayers—were assessed. **Figure 28C** illustrates the effect of MSDK on ERK5 expression in osteoblasts grown as transwell co-cultures. As shown, hMSCs exposed to Os<sup>+</sup> media alone decreased ERK5 activity (pERK5) ( $p < .001$  vs. Os<sup>-</sup>/MSDK), which was not due to decreases in total ERK5 (tERK5) levels. The addition of MSDK to the Os<sup>+</sup> media produced an increase in pERK5 relative to Os<sup>+</sup>/Veh ( $p < .05$  vs. Os<sup>+</sup>/Veh); however, this may be due to decreases in tERK5 since levels decreased when compared to Os<sup>-</sup>/MSDK ( $p < .01$  vs. Os<sup>-</sup>/MSDK). This finding is consistent with the role of ERK5 in osteoblasts ([Li, Ma et al. 2012](#), [Kaneshiro, Otsuki et al. 2015](#), [Bo, Bin et al. 2016](#)). Because ERK5 is a prosurvival kinase, decreases in its expression following Os<sup>+</sup>/Veh or Os<sup>+</sup>/MSDK exposure occurs to possibly allow for hMSCs to switch from a proliferative state towards a differentiation state as shown in **Figure 25**. Interestingly, when hMSCs were cultured in direct contact with hPBMCs (layered), no changes in pERK5 or tERK5 occurred (**Figure 28E**). Perhaps, MSDK exerted variable effects on pERK5 expression in hPBMCs and hMSCs resulting in a net zero effect because layered co-cultures contain both osteoblasts and osteoclasts. This idea is supported in **Figure 28D** demonstrating a trend ( $p = 0.19$ ) towards an increase in transwell osteoclastic pERK5 in response to MSDK. Another alternative could be that the presence of osteoclasts exert an inhibitory influence over osteoblastic pERK5 because when hMSCs were



grown in the absence of osteoclasts or as mono-cultures, MSDK increased both pERK5 and tERK5 (Figure 28F).



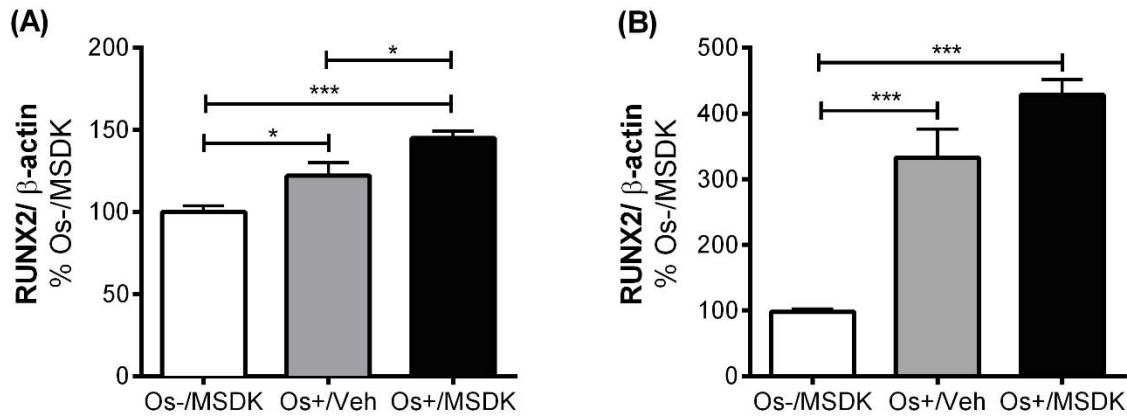


**Figure 28. Effect of MSDK on MAPK (ERK1/2 and ERK5).** After 21 days of MSDK exposure, western blot was performed to determine **(A)** ERK1/2 expression of osteoblasts grown as a transwell co-culture, **(B)** ERK1/2 expression of osteoblasts and osteoclasts grown as a layered co-culture, **(C)** ERK5 expression of osteoblasts grown as a transwell co-culture, **(D)** phospho-ERK5 expression of osteoclasts grown as a transwell co-culture, **(E)** ERK5 expression of osteoblasts and osteoclasts grown as a layered co-culture, and **(F)** ERK5 expression of osteoblasts grown as hMSC mono-cultures. Cell lysates were prepared on day 21 from the bottom (osteoblasts) or top (osteoclasts) chambers of transwells and from the whole plate (both osteoblasts and osteoclasts) in the layered co-culture. Protein levels were normalized against  $\beta$ -actin and against Os-/MSDK. Each bar represents the mean ( $\pm$  S.E.M.) expression of **(i)** phospho-ERK: total-ERK, **(ii)** phospho-ERK and **(iii)** total-ERK for ERK1/2 and ERK5 in each respective co-culture.  $*=p<.05$ ,  $**=p<.01$  and  $***=p<.001$ ; One-way ANOVA followed by Bonferroni's post-hoc multiple comparison *t*-test ( $n=6$  per group).

In summary, MSDK treatment for 21 days significantly enhanced ERK1/2 activation in osteoblasts grown as transwell co-cultures primarily by decreasing total ERK1/2. Although a similar pattern of ERK1/2 expression was observed in layered co-cultures, no such conclusion could be drawn as both osteoblasts and osteoclasts were present together in this co-culture. MSDK decreased total ERK5 expression in transwell osteoblasts and was without effect in layered co-cultures. Human MSCs grown as monolayers demonstrated increases in pERK5 and tERK5 in response to MSDK suggesting possible inhibitory influence of osteoclasts on osteoblastic ERK5 expression.

#### **3.2.4. MSDK modulates RUNX2 level in co-cultures of hMSCs and hPBMCs dependent upon the type of culturing condition—layered or transwell**

MSDK effects on runt-related transcription factor 2 (RUNX2) was examined because RUNX2 is a master regulator of osteogenesis ([Ge, Xiao et al. 2007](#), [Ge, Xiao et al. 2009](#)) and it is regulated by MAPKs ([Ge, Xiao et al. 2007](#), [Ge, Xiao et al. 2009](#)). Melatonin induces RUNX2 expression in osteoblasts differentiated from hMSCs ([Sethi, Radio et al. 2010](#)) and in bone ([Koyama, Nakade et al. 2002](#), [Witt-Enderby, Slater et al. 2012](#)). Like many of the other proteins studied, the type of co-culture dictated their response to MSDK. For example, MSDK enhanced RUNX2 expression in transwell osteoblasts beyond that induced by Os<sup>+</sup>/Veh ( $p < .05$ ) (**Figure 29A**). However, in layered osteoblasts, MSDK did not further enhance RUNX2 expression induced by Os<sup>+</sup>/Veh (**Figure 29B**). The latter may be due to the possibility that maximal levels of RUNX2 expression may already have been attained.

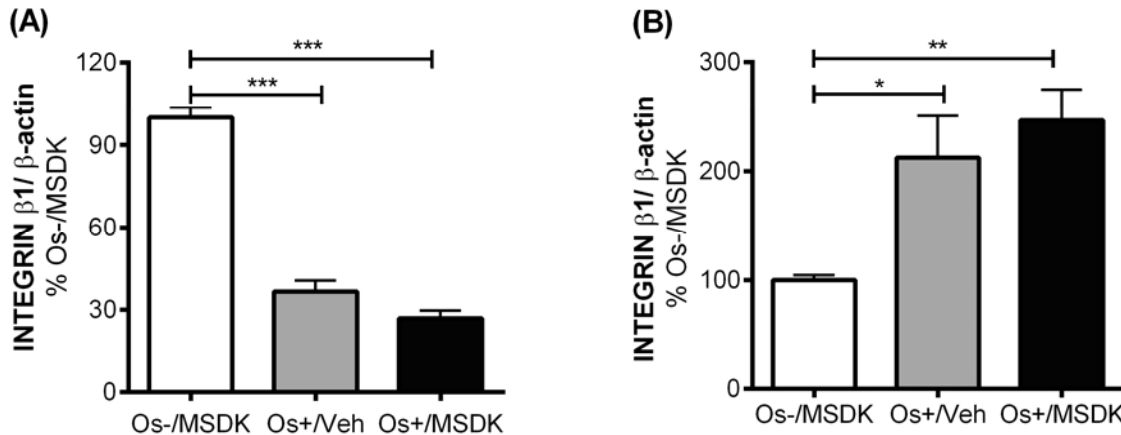


**Figure 29. Effect of MSDK on RUNX2.** After 21 days of MSDK treatment, western blot was performed to determine RUNX2 expression of osteoblasts grown in (A) transwell co-cultures and (B) layered co-culture, respectively. Cell lysates were prepared on day 21 from the bottom chamber (osteoblasts) in the transwell co-culture and from the whole plate containing both osteoblasts and osteoclasts (layered co-culture). Protein levels in each co-culture was analyzed, normalized against β-actin followed by normalization against Os-/MSDK and represented as mean (± S.E.M.). \*= $p < .05$ , \*\*= $p < .01$  and \*\*\*= $p < .001$ ; One-way ANOVA followed by Bonferroni's post-hoc multiple comparison  $t$ -test (n=6 per group).

### 3.2.5. MSDK did not modulate INTEGRIN β1 level in co-cultures of hMSCs and hPBMCs

Integrins regulate the interaction between bone cells and the extracellular matrix and thus control different aspects of bone cell growth and activity ([Mizuno, Fujisawa et al. 2000](#)). The use of these two different co-culture models permitted exploration of the roles of MSDK on this important class of cell matrix proteins, especially in the layered co-culture where osteoblasts and osteoclasts are in direct contact with each other during their differentiation. As shown in **Figure 30C and D**, the effect of Os+ media alone on INTEGRIN β1 expression was opposite in effect depending on the type of co-culture; Os+/Veh decreased INTEGRIN β1 level in transwell

osteoblasts (**Figure 30A**) but increased in layer co-culture where both cells are present. (**Figure 30B**). The addition of MSDK to Os+ did not further decrease or increase INTEGRIN  $\beta$ 1 levels in either co-culture.

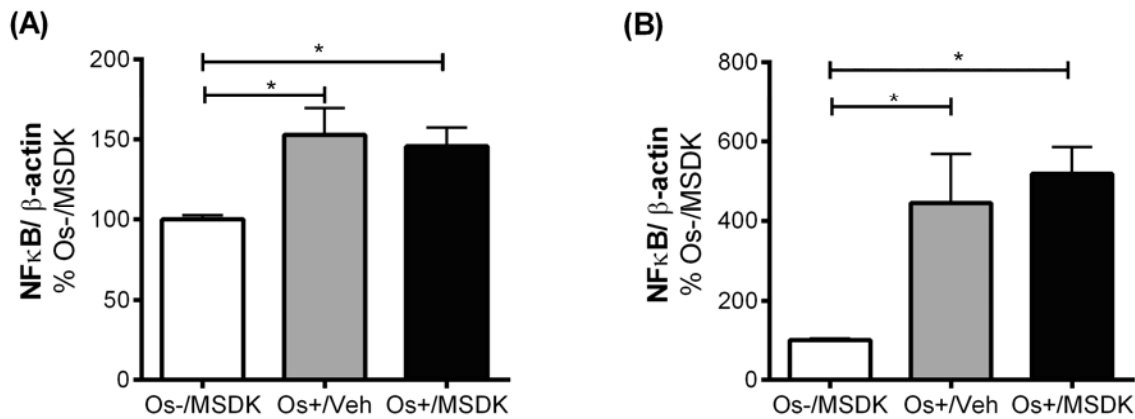


**Figure 30. Effect of MSDK on INTEGRIN  $\beta$ 1.** After 21 days of MSDK treatment, western blot was performed to determine INTEGRIN  $\beta$ 1 expression of osteoblasts grown in (A) transwell co-cultures and (B) layered co-cultures, respectively. Cell lysates were prepared on day 21 from the bottom chamber (osteoblasts) in the transwell co-culture and from the whole plate of the layered co-culture. Protein levels in each co-culture was analyzed, normalized against  $\beta$ -actin followed by normalization against Os-/MSDK and represented as mean ( $\pm$  S.E.M.). \*= $p$ <.05, \*\*= $p$ <.01 and \*\*\*= $p$ <.001; One-way ANOVA followed by Bonferroni's post-hoc multiple comparison  $t$ -test (n=6 per group).

### 3.2.6. MSDK modulates NF $\kappa$ B level in co-cultures of hMSCs and hPBMCs dependent upon the type of culturing condition—layered or transwell

Nuclear factor kappa-B (NF $\kappa$ B) plays a vital role in RANK-mediated osteoclastogenesis ([Jimi, Aoki et al. 2004](#), [Wada, Nakashima et al. 2006](#), [Boyce and Xing 2008](#)). Similarly, novel roles for NF $\kappa$ B in osteoblasts are also emerging ([Chang, Wang et al. 2009](#), [Marie 2015](#)). Therefore,

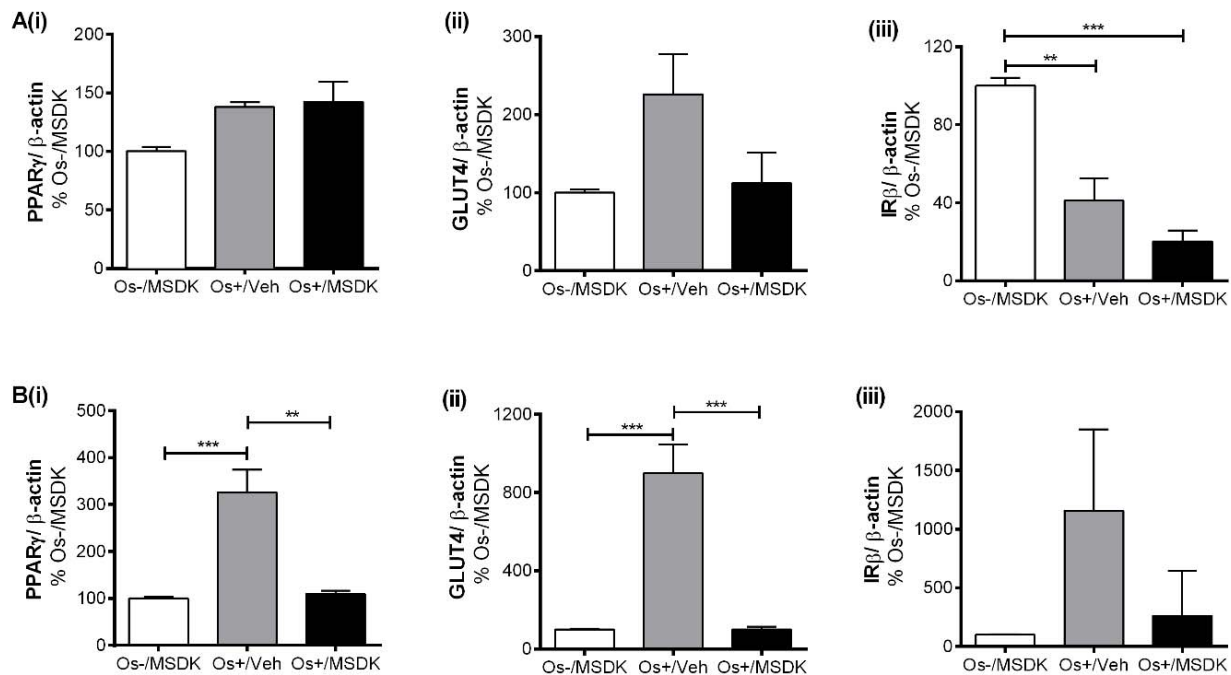
levels of NFκB in response to MSDK were assessed in transwell as well as in layered co-cultures. As shown in **Figure 31A**, hPBMCs grown as transwells with hMSCs and exposed to Os+ media containing MSDK demonstrated significant increases in NFκB vs. Os-/MSDK and Os+/Veh ( $p < .05$  vs. Os+/Veh); no increases in NFκB occurred in the presence of osteogenic media (Os+/Veh) alone. In contrast, exposure to Os+/Veh increased NFκB levels in layered co-cultures and no further enhancement occurred in presence of MSDK (**Figure 31B**). The effects of Os+/MSDK on NFκB levels in transwell osteoclasts are not easily explained considering that this same culture condition (transwell and Os+/MSDK) decreased osteoclastogenesis (**Figure 26A**).



**Figure 31. Effect of MSDK on NFκB.** After 21 days of MSDK treatment, western blot was performed to determine NFκB expression of osteoclasts grown in (A) transwell co-cultures, and (B) layered co-cultures, respectively. Cell lysates were prepared on day 21 from the top chamber (osteoclasts) in the transwell co-culture and from the whole plate containing both osteoblasts and osteoclasts (layered co-culture). Protein levels in each co-culture was analyzed, normalized against β-actin followed by normalization against Os-/MSDK and represented as mean (± S.E.M.). \*= $p < .05$ , \*\*= $p < .01$  and \*\*\*= $p < .001$ ; One-way ANOVA followed by Bonferroni's post-hoc multiple comparison  $t$ -test ( $n=6$  per group).

### 3.2.7. MSDK modulates PPAR $\gamma$ and GLUT4 levels in co-cultures of hMSCs and hPBMCs dependent upon the type of culturing condition—layered or transwell

The effects of MSDK on different metabolic proteins such as peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), glucose transporter type 4 (GLUT4) and beta subunit of insulin receptor (IR $\beta$ ) were evaluated due to their potential impact on bone cell differentiation and activity ([Akune, Ohba et al. 2004](#), [Takada, Suzawa et al. 2007](#), [Ferron, Wei et al. 2010](#), [Li, Leslie et al. 2013](#)). It was in these proteins where the type of culturing condition played vital roles in their expression in osteoblasts exposed to MSDK. For example, the addition of MSDK did not affect PPAR $\gamma$  expression in osteoblasts grown as transwell co-cultures (**Figure 32Ai**). In contrast, Os<sup>+</sup>/MSDK significantly reduced total PPAR $\gamma$  expression in osteoblasts and osteoclasts vs. Os<sup>+</sup> alone ( $p < .01$ ) in layered co-cultures (**Figure 32Bi**). Similarly, MSDK inhibited Os<sup>+</sup>/Veh-mediated GLUT4 levels in layered osteoblasts (**Figure 32Bii**), but not in transwell osteoblasts (**Figure 32Aii**). IR $\beta$  levels, though, were only modulated in transwell osteoblasts exposed to Os<sup>+</sup> media alone (**Figure 32Aiii**). No effect on IR $\beta$  levels occurred in layered osteoblasts in response to any of the treatments (**Figure 32Biii**).



**Figure 32. Effect of MSDK on metabolic proteins.** After 21 days of MSDK treatment, expression of metabolic proteins such as (i) PPAR $\gamma$ , (ii) GLUT4 and (iii) IR $\beta$  were measured in (A) osteoblasts grown in transwell co-cultures and (B) osteoblasts and osteoclasts grown in layered co-cultures. Cell lysates were prepared on day 21 from the bottom (osteoblasts) and top (osteoclasts) chambers in the transwell co-culture and from the whole plate (both osteoblast and osteoclast) in the layered co-culture. Protein levels were normalized against  $\beta$ -actin and then to Os-/MSDK. Mean protein levels were analyzed and compared between groups (Os-/MSDK, Os+/Veh, Os+/MSDK). \*= $p$ <.05, \*\*= $p$ <.01 and \*\*\*= $p$ <.001; One-way ANOVA followed by Bonferroni's post-hoc multiple comparison  $t$ -test (n=6 per group).

### 3.3. Discussion

The *in vitro* study was developed as part of the translational study to evaluate the mechanism(s) underlying the clinical effects of MSDK therapy on bone formation and bone



marker turnover in postmenopausal osteopenic women shown in the MOTS. These *in vitro* studies expanded upon the outcomes of the MOTS with a focus on identifying if and how osteoblasts modulate osteoclast activity using two novel co-culture systems consisting of human adult mesenchymal stem cells (hMSCs) as osteoblast precursors and human peripheral blood monocytes (hPBMCs) as osteoclast precursors—layered or transwell. This was intended to model the different ways osteoblasts and osteoclasts interact and communicate *in vivo* and to get information about how MSDK affects bone cells in their undifferentiated state rather than in already differentiated state. To the best of our knowledge, this is the first study assessing if and how combination of melatonin, strontium citrate, vitamin D<sub>3</sub> and vitamin K<sub>2</sub> regulate the formation and activity of osteoblasts and osteoclasts in a way that favors bone formation. This preliminary study focused only on the pathways that could potentially be regulated by MSDK, including the OPG/RANKL pathway, MAPK, RUNX2, NFκB and INTEGRIN β1 as well as on certain metabolic parameters that affect bone function, including PPARγ and GLUT4. Deep exploration into these pathways is required to obtain a full picture of the underlying mechanism of MSDK's modulatory activity on bone.

The complex relationship between osteoblasts and osteoclasts and their regulation of each other's activity during the bone remodeling process are still poorly understood. Bone remodeling intrinsically depends on multiple modes of communication that occur between osteoblastic and osteoclastic lineage cells at various stages of their differentiation. These include: direct cell-to-cell contact to allow for the interaction between membrane-bound ligands and the initiation of intracellular signaling; or via the release of diffusible paracrine factors such as growth factors, cytokines, chemokines and other small molecules from either cell type to control each other's activity; or by forming gap junctions through which small molecules can pass between the two

cells ([Matsuo and Irie 2008](#), [Sims and Gooi 2008](#)). In the present study, the layered co-culture allows for direct contact between osteoblasts and osteoclasts, whereas the transwell co-culture is based on indirect contact through the release of paracrine factors from either cell. By mimicking more closely the *in vivo* environment, these co-cultures systems were advantageous to evaluate how MSDK modulates the intercellular communication between bone cells throughout their transition from the immature to mature stage of life.

Calcium deposition and bone mineralization by mature osteoblasts is an important marker of bone formation. The 21-day time period was chosen based on past studies, which showed that continuous melatonin treatment for 21 days is required to induce osteoblast differentiation and mineralization from hMSCs, as revealed by alkaline phosphatase (ALP) activity and calcium deposition (alizarin red staining) assay ([Sethi, Radio et al. 2010](#)). Multi-potent human bone marrow stromal cells are the primary osteoblast lineage cells, which were found to differentiate into pre-osteoblast in 10 days when exposed to 50nM melatonin daily, as revealed by ALP activity ([Radio, Doctor et al. 2006](#)). Osteogenic medium containing ascorbate, dexamethasone and  $\beta$ -glycerophosphate was required to regulate hMSC differentiation into pre-osteoblasts and mature osteoblasts, as basal growth media only induces hMSC proliferation ([Radio, Doctor et al. 2006](#), [Sethi, Radio et al. 2010](#), [Langenbach and Handschel 2013](#)). Pre-osteoblasts proliferate and start to differentiate into mature osteoblasts, which then begin to deposit calcium in the bone matrix. Usually *in vitro* mineralization occurs between 14 to 12 days ([Sethi, Radio et al. 2010](#)) and so the same was expected to occur in the present study. Therefore, hMSCs were cultured for 13 days so that they would mature into pre-osteoblasts and begin secreting RANKL and M-CSF. This was to facilitate osteoclastogenesis after peripheral blood monocytes were added to the culture on day 13. By day 21, pre-osteoblasts differentiate into osteoblasts and stop osteoclastogenesis by producing

OPG ([Atkins, Kostakis et al. 2003](#), [Boyce and Xing 2008](#)). Successful differentiation of osteoblasts from hMSCs and osteoclasts from hPBMCs in vehicle-treated culture (Os+/Veh) indicated that both co-cultures were developed successfully without the external addition of RANKL and M-CSF.

The 21 days of MSDK treatment greatly induced calcium deposition activity of the differentiated osteoblasts, as revealed by alizarin red staining. Our findings regarding the mineralization effect of melatonin in both cultures is consistent with previous *in vitro* studies, which show stimulatory roles of melatonin in osteoblast differentiation and mineralization from hMSCs and pre-osteoblasts ([Radio, Doctor et al. 2006](#), [Zaminy, Ragerdi Kashani et al. 2008](#), [Sethi, Radio et al. 2010](#), [Zhang, Su et al. 2010](#), [Park, Kang et al. 2011](#), [Maria and Witt-Enderby 2014](#)). *In vitro* bone-forming effects of melatonin are further supported by earlier preclinical and clinical studies ([Clafshenk, Rutkowski et al. 2012](#), [Kotlarczyk, Lassila et al. 2012](#), [Witt-Enderby, Slater et al. 2012](#)).

Numerous *in vitro* studies show that strontium ranelate stimulates osteogenic differentiation of MSCs and pre-osteoblasts ([Atkins, Welldon et al. 2009](#), [Fromigué, Hay et al. 2009](#), [Peng, Zhou et al. 2009](#)); rebalances bone marrow osteoblastogenesis and adipogenesis ([Saidak, Hay et al. 2012](#)); and increases osteoblast maturation, matrix mineralization and bone nodule numbers in osteoblast cultures ([Bonnelye, Chabadel et al. 2008](#), [Atkins, Welldon et al. 2009](#), [Querido and Farina 2013](#)). Similar to published studies, strontium citrate alone in the MOTS enhanced osteoblast differentiation and mineralization from hMSCs after 21 days of exposure in the layered co-culture.

Published studies show an *in vitro* stimulatory effect of vitamin K<sub>2</sub> (MK7) on the post-proliferative stages of osteoblast differentiation and bone formation (i.e., the osteoblast to

osteocyte transition) via a  $\gamma$ -carboxylation-dependent and independent mechanism ([Yamaguchi, Sugimoto et al. 2001](#), [Katsuyama, Otsuki et al. 2005](#), [Atkins, Welldon et al. 2009](#), [Yamaguchi and Weitzmann 2011](#)). MK7 triggers osteocalcin protein expression ([Yamaguchi and Weitzmann 2011](#)) and induces the expression of osteogenic genes [e.g., growth differentiation factor 10 (*GDF10*), insulin-like growth factor 1 (*IGF1*), and vascular endothelial growth factors (*VEGFA*)] ([Gigante, Brugè et al. 2015](#)). However, in the present study, no effect of MK7 alone on osteoblast mineralization occurred in either co-culture. This could be due to the lower treatment concentration of MK7 used in present study (18.5 nM) as compared to the concentrations used in past-published studies ( $10^{-7}$  to  $10^{-5}$  M) ([Yamaguchi, Sugimoto et al. 2001](#), [Atkins, Welldon et al. 2009](#), [Yamaguchi and Weitzmann 2011](#), [Gigante, Brugè et al. 2015](#)) or that the treatment duration was not long enough to allow for the osteoblast-osteocyte transition.

The stimulatory effect of vitamin D<sub>3</sub> on active calcium and phosphate absorption and uptake into bone and overall homeostasis is well-established ([Lips 2006](#)) and past studies demonstrate a complex relationship between vitamin D and osteoblasts. In our study, no effect of vitamin D<sub>3</sub> alone on osteoblast differentiation occurred in either co-culture, even though other studies demonstrate the presence of the vitamin D receptor (VDR) and the protein disulfide isomerase family A member 3 receptor (Pdia3R) ([Chen, Dosier et al. 2013](#)) in osteoblasts, which allows for the direct action of vitamin D<sub>3</sub> on osteoblast differentiation, proliferation and mineralization ([Anderson, Lam et al. 2013](#), [Yang, Atkins et al. 2013](#)). Vitamin D<sub>3</sub> inhibits osteogenic genes such as *COL1A1* and *ALP* expression in pre-osteoblasts and enhances their expression in the late differentiation stage in osteoblasts. The effect of vitamin D<sub>3</sub> during the later stage of bone formation is also demonstrated by inducing the osteoblast to osteocyte transition and regulation of key genes such as *FGF23* and *DMPI* that stimulates osteocyte mineralization

([Anderson, Lam et al. 2013](#), [Kogawa, Findlay et al. 2013](#)). However, for vitamin D<sub>3</sub>, the magnitude of its osteogenic effect depends on the duration of exposure, dosage as well as the origin and maturation stage of osteoblasts ([Anderson, Lam et al. 2013](#), [Yang, Atkins et al. 2013](#)). Perhaps, our study conditions were different than others' in these aspects and explains why no increases in calcium deposition following exposure to vitamin D<sub>3</sub> occurred in the MOTS. Another explanation could be that combination of vitamin D<sub>3</sub> with other factors like MK7 (vitamin K<sub>2</sub>) may increase vitamin D<sub>3</sub>'s effects. This idea is supported by Gigante *et al.* who rationalized that co-supplementation with MK7 enhances vitamin D<sub>3</sub>- regulated osteogenic gene expression and differentiation of human MSCs. MK7 also maintains an optimum balance between the induction and carboxylation of osteocalcin, required for its action on the extracellular matrix (ECM) ([Gigante, Brugè et al. 2015](#)). These data further support our findings where the individual compounds showed little or no significant mineralization effect; however, when combined, their effects maximized even when they were used at their lowest concentrations.

The role of these compounds on osteoclast formation and activity is emerging. Little evidence is available on melatonin's effect on osteoclasts; however, others have shown that melatonin produces an inhibitory effect on RANKL-mediated osteoclastogenesis perhaps due to increases in OPG mRNA and protein expression in osteoblasts ([Koyama, Nakade et al. 2002](#), [Maria and Witt-Enderby 2014](#)). These melatonin-mediated increases in OPG would block osteoclastogenesis by acting as a RANKL decoy receptor ([Gasser and Kneissel 2017](#)).

Strontium ranelate prevents osteoclast differentiation via the OPG/RANKL/RANK pathway and resorption activity by disrupting the organization of the actin cytoskeleton ([Bonnelye, Chabadel et al. 2008](#), [Atkins, Welldon et al. 2009](#), [Saidak and Marie 2012](#)). Regarding strontium citrate, no such data are available regarding its effects on osteoclasts and MK7 negatively regulates

osteoclast survival and activity by inhibiting RANKL-mediated NF $\kappa$ B activation ([Yamaguchi and Weitzmann 2011](#)).

Vitamin D<sub>3</sub>, in contrast, stimulates osteoclast differentiation from monocytes ([Bar-Shavit, Teitelbaum et al. 1983](#)) and directly regulates bone resorption by activating human RANKL genes in osteoblasts through vitamin D responsive elements (VDREs) ([Kitazawa, Kajimoto et al. 2003](#)). These effects of vitamin D occur by increasing RANKL and decreasing OPG expression on osteoblasts and stromal cells ([Kogawa, Findlay et al. 2010](#)). Circulating levels of vitamin D<sub>3</sub> precursors [25(OH)D<sub>3</sub>] and its metabolism to 1,25(OH)<sub>2</sub>D<sub>3</sub> by osteoclast precursors are important regulators that optimize osteoclast differentiation via effects on gene expression and by promoting the coupling of bone resorption to formation ([Kogawa, Findlay et al. 2010](#), [Anderson, Lam et al. 2013](#)).

The effect of MSDK on osteoclast differentiation and function was measured by TRAP because TRAP is a well-known marker of terminally differentiated osteoclasts ([Angel, Walsh et al. 2000](#), [Halleen, Tiiinen et al. 2006](#)). Osteoclast differentiation was inhibited following treatment with MSDK-supplemented osteogenic media in transwell co-cultures, but not in layered co-cultures even though MSDK inhibited resorption pit formation activity in layered co-cultures. Osteoclasts, grown as layered co-cultures, were strongly inhibited in the presence of osteogenic (Os+) medium alone and in combination with MSDK. This could be due to the direct cell-to-cell contact or by release of paracrine factors between differentiating osteoblasts and osteoclasts leading to co-regulation of each other's state of differentiation and activity in these two co-culture systems.

The discrepancy in the robustness of MSDK's effects on osteoblast and osteoclast differentiation, as observed between the culturing conditions (i.e., greater increase in transwell

osteoblast differentiation vs. layered; greater decrease in layered osteoclast differentiation vs. transwell), could be explained by the types of signaling pathways activated in both bone cells. For example, osteoblast formation may be favored in both co-cultures; however, the mechanisms may be very different especially in how osteoclasts modulate osteoblast differentiation. In transwell co-cultures, paracrine factors (e.g., IGF I and II, FGF, TGF 1 and 2, BMPs 2, 3, 4, 6 and 7 and PDGF) released by osteoclasts may favor osteoblast formation through these distinct signaling cascades whereas in the layered co-culture model, with both cells in direct contact, osteoclasts may mediate osteoblast differentiation through the contact-dependent ephrin signaling pathway. The contact-dependent ephrin signaling pathway can, in turn, negatively regulate osteoclastogenesis irrespective of their resorbing activity. As ephrin signaling requires close contact between osteoclasts and osteoblasts, this inhibition was not observed in the transwell co-culture. Another mechanism to explain these differences could be due to osteoblast-derived osteoclast inhibitory lectin (OCIL), which is a type II transmembrane C-type lectin, that can suppress both osteoclast differentiation ([Kartsogiannis, Sims et al. 2008](#), [Matsuo and Irie 2008](#)) and osteoblast differentiation and function *in vitro* ([Nakamura, Ly et al. 2007](#), [Matsuo and Irie 2008](#)); this pathway helps to maintain normal bone physiology. In our layered co-culture model, the cell-to-cell contact between osteoblasts and osteoclasts may stimulate OCIL to inhibit the differentiation of osteoblasts, osteoclasts or both resulting in an overall diminished state of differentiation.

Another possibility could be explained by the presence of vitamin D<sub>3</sub>, which can stimulate osteoclastogenesis from monocytes ([Bar-Shavit, Teitelbaum et al. 1983](#)). Therefore, even if the other components of MSDK (i.e., melatonin, strontium citrate and MK7) are inhibiting osteoclastogenesis, some of these effects may be masked by vitamin D<sub>3</sub>'s stimulatory effects on osteoclast differentiation. These data suggest that MSDK neither completely inhibited bone

resorption like other conventional therapies, nor only assisted bone formation. Rather, it may be working to switch the balance between bone-forming osteoblasts and bone-resorbing osteoclasts towards equilibrium to maintain healthy bone remodeling. These data are consistent with the findings of our MOTS clinical trial where postmenopausal osteopenic women taking MSDK supplementation over one year had rebalanced serum bone marker (CTx:P1NP) turnover where increases in serum P1NP was observed in the MSDK group, while steady levels of CTx were maintained to keep bone remodeling (i.e. osteoblast:osteoclast ratios) balanced.

MSDK triggered osteoblasts to produce more OPG and less RANKL in the transwell co-cultures even though the individual components (i.e., melatonin, strontium citrate, vitamin D<sub>3</sub> and K<sub>2</sub>) produced variable effects on OPG and RANKL. Melatonin and strontium ranelate positively regulate OPG and negatively regulate RANKL in osteoblasts ([Koyama, Nakade et al. 2002](#), [Atkins, Welldon et al. 2009](#), [Saidak and Marie 2012](#)) while vitamin K<sub>2</sub> increases the expression of both OPG and RANKL in osteoblastic MC3T3E1 cells ([Katsuyama, Otsuki et al. 2005](#)). The relative expression of OPG and RANKL in osteoblasts is a critical transition point for balancing bone mineralization ([Boyce and Xing 2008](#)). MSDK was more likely balancing osteoblast and osteoclast activities initially through its differentiating effects on osteoblasts; these mature osteoblasts then begin to express OPG to modulate RANKL-mediated osteoclastogenesis to maintain appropriate bone remodeling. This concept is corroborated in the MOTS demonstrating a steady level of CTx throughout MSDK treatment. This is also consistent with what was observed for RANKL expression in osteoblasts in response to MSDK, that is, levels were significantly decreased in response to the osteogenic media (Os<sup>+</sup>) and remained low even in the presence of MSDK. Because RANKL production is largely regulated by immature osteoblasts ([Atkins, Kostakis et al. 2003](#)),



maturation of osteoblasts in response to MSDK would result in less RANKL production as observed in our study.

Besides being reduced as osteoblasts mature, RANKL can go through various stages of processing to promote osteoclast activation which is generated from the ectodomain shedding of membrane-bound RANKL via the action of matrix metalloproteases 3, 7 and 14 and ADAM 10, 17 (or TACE) and 19 ([Nakashima, Kobayashi et al. 2000](#), [Wada, Nakashima et al. 2006](#)). In our study, the addition of MSDK in osteogenic (Os<sup>+</sup>) medium decreased the 25kDa RANKL peptide fragment in transwell co-culture but not in layered co-culture; this transwell effect of MSDK is indicative of a facilitation of ADAM 10's catalytic activity ([Hikita, Yana et al. 2006](#)). In the transwell co-cultures, formation of the 24kDa RANKL peptide was inhibited in presence of osteogenic (Os<sup>+</sup>) medium alone; no further decrease was observed with MSDK. The modulation of the 24kDa fragment by Os<sup>+</sup> in transwell osteoblasts indicates that MMP 14 may be playing some type of role in osteoblastogenesis and osteoclastogenesis ([Hikita, Yana et al. 2006](#)).

The addition of MSDK did not further affect the levels of secreted OPG and RANKL as measured in the cell culture media. In fact, osteoblastic differentiation and maturation correlated more with an increase in secreted RANKL than with secreted OPG. Similar effects were observed with parathyroid hormone (PTH)—the only bone anabolic agent currently available—which increases sRANKL secretion from MC3T3-E1 cells, while not affecting sOPG, resulting in reduced sOPG: sRANKL ratios ([Coetzee, Haag et al. 2007](#)).

The role of membrane-bound and secreted RANKL in osteoclastogenesis has not been fully characterized. Nevertheless, studies show that the membrane-bound form of RANKL is more efficient at inducing osteoclastogenesis than the soluble form ([Nakashima, Kobayashi et al. 2000](#)). Therefore, the inhibitory effect of MSDK on osteoclastogenesis could possibly be attributed by

the regulation of membrane bound OPG and RANKL, rather than secreted OPG and RANKL. This, however, does not explain MSDK's effects on osteoclastogenesis in the layered co-cultures. Other variables like direct interference of osteoclasts on osteoblast functionality may play a role because a less robust increase in osteoblast differentiation by MSDK in layered co-culture may produce a less robust change in membrane-bound OPG and RANKL. This is supported in the mono-culture studies where hMSCs grown in the absence of osteoclasts had robust increases in both OPG and RANKL.

Osteoblast differentiation and proliferation involve multiple signaling pathways ([Rauci, Bellosta et al. 2008](#), [Maria and Witt-Enderby 2014](#)). The present study focused on the effect of MSDK on MAPKs, particularly ERK1/2 and ERK5, in modulating bone cell physiology. ERK1/2 are one of the key regulatory proteins involved in osteoblast differentiation ([Ge, Xiao et al. 2007](#), [Ge, Xiao et al. 2009](#), [Matsushita, Chan et al. 2009](#), [Greenblatt, Shim et al. 2013](#)) and thus play essential roles in bone remodeling. Increased ERK1/2 signaling switches mesenchymal cell differentiation towards osteoblasts from chondrocytes and vice-versa. Furthermore, specific deletion of ERK1 and ERK2 in mouse limb mesenchyme results in low bone mineralization ([Matsushita, Chan et al. 2009](#)). ERK also regulates ATF4, which is a late stage mediator of osteoblast differentiation ([Greenblatt, Shim et al. 2013](#)). In the present study, MSDK treatment enhanced ERK1/2 activation by downregulating total ERK1/2 levels in both co-culture models—transwell and layered. ERK1/2 is also expressed in osteoclasts and plays a vital role in osteoclast differentiation where high ERK1/2 is associated with increases in osteoclast activity ([Matsushita, Chan et al. 2009](#)). In layered co-cultures, which contain both osteoblastic and osteoclastic ERK1/2, MSDK may have increased pERK1/2 levels in both cells resulting in an increase in osteoblastogenesis and a decrease in osteoclastogenesis. It is more likely that MSDK increased

osteoblastic pERK1/2 because MSDK under these conditions inhibited osteoclasts. Previous *in vitro* studies in our lab ([Radio, Doctor et al. 2006](#), [Sethi, Radio et al. 2010](#)) and others' ([Zhang, Su et al. 2010](#), [Park, Kang et al. 2011](#)) show that melatonin, through the activation of MT2 melatonin receptors on hMSC monolayers, triggers ERK1/2 signaling ([Radio, Doctor et al. 2006](#)); this leads to increases in osteogenic gene expression such as *RUNX2*, *BMP2* and *OC* resulting in osteoblastogenesis ([Radio, Doctor et al. 2006](#), [Sethi, Radio et al. 2010](#)). Ge *et al* showed that *Runx2*<sup>+/-</sup> mice expressing constitutively active and dominant-negative mutants of MAPK in their osteoblasts exhibit low clavicular and calvarial bone mass and hypomineralization ([Ge, Xiao et al. 2007](#)). The bone mass is restored by breeding *Runx2*<sup>+/-</sup> mice with a constitutively active mutant of the MEK1 transgene bearing mice ([Ge, Xiao et al. 2009](#)). These data demonstrate a direct relationship between the ERK1/2 pathway and RUNX2 ([Ge, Xiao et al. 2007](#), [Ge, Xiao et al. 2009](#)). Strontium ranelate also enhances *Runx2* expression in murine osteoblasts via the RAS/ERK 1/2 MAPK signaling pathway ([Peng, Zhou et al. 2009](#)). Vitamin K<sub>2</sub>, at a 10 μM dose, induces *RUNX2/Runx2* and *OSTERIX/Osterix* expressions in primary bone marrow stromal cells and MC3T3, respectively ([Yamaguchi and Weitzmann 2011](#)). The role of vitamin D<sub>3</sub> on osteogenic genes expression is species specific, as it increases *BGLAP*, *SPPI*, *RUNX2* gene expression in human primary osteoblasts while producing inhibitory effects on murine osteoblasts ([Kogawa, Findlay et al. 2010](#)).

In our study, MSDK exposure induced transwell osteoblast RUNX2 expression beyond that induced by Os<sup>+</sup> alone; however, in layered co-cultures, MSDK produced no additional increases in RUNX2 expression. Based on previous studies ([Radio, Doctor et al. 2006](#), [Sethi, Radio et al. 2010](#)) and the present study, we propose that the MSDK-mediated increases in RUNX2 expression and osteoblast differentiation from hMSCs in these co-cultures is occurring, in part, via

the ERK1/2 pathway. Because other pathways, such as p38, JNK, BMPs, canonical Wnt, also regulate *RUNX2* transcriptional activity ([Franceschi and Xiao 2003](#), [Gaur, Lengner et al. 2005](#), [Rodríguez-Carballo, Gámez et al. 2016](#)), these pathways cannot be ruled out and require further investigation.

Even though the role of ERK1/2 in osteoblastic differentiation has been extensively studied, little is known about the role of ERK5 in osteoblast physiology. ERK5 is well known for regulating various cellular processes including proliferation, differentiation, transformation and survival in certain cells types under certain conditions; however, most studies focused on its role in cardiovascular development and neuronal differentiation ([Nishimoto and Nishida 2006](#), [Drew, Burow et al. 2012](#)). Some studies have shown an involvement of ERK5 in fluid shear stress-mediated cell proliferation in osteoblasts ([Li, Ma et al. 2012](#), [Bo, Bin et al. 2016](#)). *Kaneshiro et al* recently demonstrated that the MEK5/ERK5 suppresses osteoblast differentiation, but promotes osteoblastic cell proliferation in pre-osteoblastic MC3T3-E1 and bone marrow stromal cells ([Kaneshiro, Otsuki et al. 2015](#)). This is consistent with what was seen in our hMSC mono-culture studies where both total and phospho-ERK5 levels increased. However, in contrast to the osteoblast mono-cultures, both total and phospho-ERK5 levels decreased in transwell osteoblasts following exposure to Os<sup>+</sup> alone and in the presence of MSDK possibly to allow for osteoblast differentiation. This is consistent with the process of osteoblast differentiation where stages of rapid cell proliferation are followed by stages of low proliferation and high differentiation. The presence of osteoclasts in this co-culture may also be playing a role on osteoblast differentiation. MSDK, added in combination with osteogenic (Os<sup>+</sup>) medium, slightly but significantly increased phospho-ERK5 levels in transwell osteoblasts perhaps to increase the number of osteoblasts before they enter a stage of differentiation.

Even though ERK5 inhibits osteoblast differentiation, it is shown to produce a stimulatory effect on osteoclast differentiation mediated via M-CSF and c-Fos induction ([Amano, Chang et al. 2015](#)). In the present study, MSDK exposure did not affect transwell osteoclast ERK5 expression even though osteoclast differentiation was decreased. Failure to inhibit the ERK5 pathway in osteoclasts supports the fact that ERK5 in transwell osteoclasts may not be involved in MSDK-mediated inhibition of osteoclastogenesis; however, this can only be verified by use of MEK5-specific inhibitors. The lack of an MSDK effect on ERK5 expression in layered co-cultures may be attributed to the fact that both osteoblasts and osteoclasts express ERK5; MSDK may be producing variable effects on ERK5 in osteoblasts and osteoclasts masking any increases or decreases that may have occurred individually in the cells. Further downstream pathways as well as related transcription factors are needed to be evaluated to get a clearer idea about the involvement of ERK1/2 and ERK5 pathways in MSDK-mediated modulation of bone cell activity.

Integrin-mediated cell–cell and cell–matrix interactions (via cytoskeletal organization and signal transduction) are key requirements for bone cell proliferation, differentiation, migration and apoptosis as well as skeletal development and homeostasis ([Clover, Dodds et al. 1992](#), [Gronthos, Stewart et al. 1997](#), [Horton and Helfrich 2000](#)). Osteoblasts primarily express  $\beta 1$  and  $\beta 5$  integrins, depending on the type of osteoblast lineage, stage of differentiation, and culture conditions employed. Usually, differentiated osteoblasts have increased expression of fibronectin receptor  $\alpha 5\beta 1$  followed by collagen binding integrin  $\alpha 2\beta 1$  expression but at a much lower level ([Horton and Helfrich 2000](#)). In the present study, INTEGRIN  $\beta 1$  expression in transwell osteoblasts was decreased in response to osteogenic (Os<sup>+</sup>) medium, which remained low even in the presence of MSDK. In layered co-cultures, however, exposure to Os<sup>+</sup> medium increased INTEGRIN  $\beta 1$  levels, which remained high when MSDK was added. These differences between co-cultures could be

explained by the fact that unlike transwell co-cultures, layered co-cultures offer a direct cell-to-cell communication for which integrin expression is required. Secondly, in the layered co-culture, levels may be higher because INTEGRIN  $\beta$ 1 levels were being measured in both the osteoblast and osteoclast; osteoclasts and their precursors express different subunits of INTEGRIN  $\beta$ 1 in order to regulate actin ring reorganization and bone resorption ([Clover, Dodds et al. 1992](#), [Helfrich, Nesbitt et al. 1996](#), [Rao, Lu et al. 2006](#)). Even though INTEGRIN  $\beta$ 1 plays an important role in osteoblast differentiation ([Horton and Helfrich 2000](#), [Mizuno, Fujisawa et al. 2000](#)), MSDK treatment showed no effect on INTEGRIN  $\beta$ 1 expression in either co-culture, suggesting that either MSDK's activity occurs via a non-integrin dependent mechanism or that the peaks and troughs observed following Os<sup>+</sup> exposure for the layered and transwell co-cultures, respectively, reached their maximums and no further effects by MSDK could be achieved.

RANKL-RANK mediated activation of the NF $\kappa$ B signaling pathway in osteoclasts plays a crucial role in osteoclast differentiation and bone resorption ([Jimi, Aoki et al. 2004](#), [Wada, Nakashima et al. 2006](#), [Boyce and Xing 2008](#)). In osteoblasts, high NF $\kappa$ B is associated with low bone mass and an inhibition of osteoblast differentiation ([Chang, Wang et al. 2009](#), [Marie 2015](#)). Strontium ranelate and vitamin K<sub>2</sub> produce suppressive effects on NF $\kappa$ B resulting in an increase in osteoblastogenesis and an inhibition of osteoclastogenesis ([Yamaguchi and Weitzmann 2011](#), [Yamaguchi and Weitzmann 2012](#)). Melatonin is found to inhibit NF $\kappa$ B as part of its anti-inflammatory effect on renal cells ([Li, Nickkholgh et al. 2009](#)). These findings prompted us to determine if MSDK's inhibitory effect on osteoclastogenesis was mediated via NF $\kappa$ B. In both transwell and layered osteoblasts, levels of NF $\kappa$ B increased in the presence of osteogenic (Os<sup>+</sup>) medium, which was not further enhanced by MSDK. These data suggest that MSDK did not induce osteoblastogenesis or inhibit osteoclastogenesis via NF $\kappa$ B signaling.

From here, we shifted our focus towards the effect of MSDK on the regulators of energy metabolism. Metabolic regulators such as peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), glucose transporter 4 (GLUT4) and insulin receptor beta (IR $\beta$ ) expressed in osteoblasts not only regulate glucose metabolism but each play an important role in osteoblast and osteoclast function ([Akune, Ohba et al. 2004](#), [Lecka-Czernik and Suva 2006](#), [Takada, Suzawa et al. 2007](#), [Ferron, Wei et al. 2010](#), [Li, Leslie et al. 2013](#)). Insulin receptor signaling in osteoblasts has been shown to play a vital role in osteoblast-mediated effects on osteoclastic bone resorption ([Ferron, Wei et al. 2010](#)). In our study, IR $\beta$  was assessed in both co-cultures—transwell and layered—and only in transwell osteoblasts was IR $\beta$  modulated. Specifically, both Os<sup>+</sup> alone or in combination with MSDK decreased IR $\beta$  in transwell osteoblasts, which was not further decreased in the presence of MSDK probably due to a bottoming effect. The decreases in IR $\beta$  in transwell osteoblasts correlate with high OPG, which is consistent with insulin's function in osteoblasts, that is, to modulate bone resorption through OPG. In osteoblasts with normal insulin signaling, bone resorption is high due to a lack of OPG expressed in the osteoblast, thus increasing RANKL-mediated osteoclast activity ([Ferron, Wei et al. 2010](#)). In our study, MSDK decreased transwell osteoblast IR $\beta$ , increased OPG, and decreased TRAP; this would explain the decrease in osteoclastogenesis in response to MSDK.

PPAR $\gamma$  is a key regulator in adipogenesis, energy expenditure, and lipid, glucose, and insulin metabolism. In bone, high levels of PPAR $\gamma$  may cause bone loss by switching the fate of mesenchymal stem cells towards adipogenesis at the expense of osteoblastogenesis ([Akune, Ohba et al. 2004](#)); this results in an increase in bone marrow fat content. A PPAR $\gamma$  insufficiency increases bone mass by enhancing osteoblastogenesis ([Akune, Ohba et al. 2004](#), [Lecka-Czernik and Suva 2006](#), [Takada, Suzawa et al. 2007](#)). PPAR $\gamma$  also supports osteoclastogenesis by stimulating

RANKL production ([Akune, Ohba et al. 2004](#), [Lecka-Czernik 2010](#)), which may further contribute to bone loss by activating osteoclasts. Melatonin and strontium ranelate directly inhibit adipogenic differentiation of hMSCs and murine MSCs, respectively, by suppressing PPAR $\gamma$  expression in favor of osteoblastogenesis ([Zhang, Su et al. 2010](#), [Saidak, Haÿ et al. 2012](#)). PPAR $\gamma$  signaling is mainly involved in the earlier stages of osteogenesis and adipogenesis and does not affect cell function ([Akune, Ohba et al. 2004](#)). Therefore, the stimulatory effect of Os<sup>+</sup> medium on PPAR $\gamma$  expression in either co-culture could be explained by the fact that when the stem cell progenitors are differentiated into osteoblasts, the osteogenic media is enhancing PPAR $\gamma$  expression to support energy metabolism. Even though MSDK addition did not affect PPAR $\gamma$  levels in the transwell co-cultures, it significantly decreased Os<sup>+</sup>-induced PPAR $\gamma$  levels in layered co-cultures. Because osteoclasts were in direct contact with osteoblasts in this culture, reduced PPAR $\gamma$  expression by MSDK could reduce the energy capabilities of osteoclasts preventing their differentiation and reducing their activity. MSDK also decreased Os<sup>+</sup>-induced GLUT4 expression in layered osteoblasts, but not in transwell osteoblasts, similar to its effect on PPAR $\gamma$ . A positive correlation between PPAR $\gamma$  and GLUT4 exists. Specifically, the PPAR $\gamma$  agonists, rosiglitazone and pioglitazone, enhance GLUT4 mRNA in diabetes mellitus type 2 (DM2) muscle tissue and a loss of PPAR $\gamma$  results in a decrease in GLUT1 and GLUT4 function in adipocytes ([Armoni, Harel et al. 2007](#), [Liao, Nguyen et al. 2007](#)). GLUT4 mRNA levels increase during osteoblast differentiation ([Li, Leslie et al. 2013](#)). In addition to GLUT4, GLUT1 also plays an important role in bone formation and is 100 fold more abundant than other glucose transporters in bone cells ([Wei, Shimazu et al. 2014](#)). Therefore, no effect of MSDK on GLUT4 could imply that a potential role of insulin independent GLUT1 pathway in MSDK's action. However, further analysis with GLUT1 is required to reach to a plausible conclusion. Osteoblastogenesis requires a significant



amount of energy, which could explain why both PPAR $\gamma$  and GLUT4 expression increased when exposed to Os<sup>+</sup> alone. Even more importantly, though, were the findings that MSDK, in combination with Os<sup>+</sup>, significantly reduced their expression consistent with MSDK's stimulatory effect on osteoblastogenesis and inhibitory effect on osteoclastogenesis.

These findings also provide a potential mechanism for the increase in bone formation and stabilization of weight in postmenopausal osteopenic women taking MSDK as shown in the MOTS; and for the improvement in femoral bone mineral density ([Amstrup, Sikjaer et al. 2015](#)) and decreases total fat mass ([Amstrup, Sikjaer et al. 2015](#)) observed in postmenopausal osteopenic women taking melatonin in the MelaOst trial. These findings were underscored by another study showing that melatonin induces osteoblast differentiation from human adipose-derived mesenchymal stem cells ([Maria, Swanson et al. 2017](#)).

## Chapter 4: Strength and limitations

Limitations to this study include low number of subjects (n=22) and lack of a diverse cohort, which made it difficult to generalize the MOTS to a larger heterogeneous population of osteopenic women. Low number of subject could be a potential reason for not getting some possible significant differences between groups, for example, a definite effect of MSDK on hip BMD could be revealed with high “n”. Besides, ethnic variation may play a role in MSDK’s effects on bone. For example, MOTS cohort only contained Caucasian women, all of which were well-educated, affluent and health conscious. Therefore, the study outcome does not necessary depict the bone loss scenario and MSDK’s effect on the women from another ethnicity or following a different lifestyle. Potential seasonal/diurnal effects may have occurred due, in part, to a revolving recruitment paradigm. This was minimized by having the participants come to the Center for Pharmacy Care at the same time of day throughout the entire year. Also, when stratified by season, no significant differences in bone marker turnover was observed in MOTS participants enrolled in the fall vs. the spring (see **Appendix VII**). Due to limited resources, we could not assess the exclusive effect of each micronutrient alone on primary and secondary endpoints. For a similar reason, bone histomorphometric analysis was not performed and so the effect of this therapy on bone microarchitecture and quality could not be assessed.

There are several strengths to this study. The translational approach allows this study to not only assess the clinical effect of MSDK in postmenopausal osteopenia, but also evaluated the underlying mechanisms governing those effects enhancing the relevance and lending support to the clinical findings. Although small, the clinical component of the MOTS was effectively designed as a double-blinded, randomized and placebo-controlled trial to avoid statistical bias. Allocation concealment was strictly followed throughout the study. Extensive inclusion and

exclusion criteria ensured a homogeneous group of study population with no competing conditions that could interfere with the outcomes. Unique to this study is that health-related QOL was assessed alongside bone health measures in this postmenopausal cohort to provide a well-balanced study assessing objective and subjective measures. The *in vitro* component of the MOTS was also constructive to assess MSDK effects at a mechanistic level and supported the clinical findings.

## Chapter 5: Conclusions

In the Melatonin-micronutrients Osteopenia Treatment Study (MOTS; NCT01870115), we investigated whether a novel combination of melatonin and three other natural bone-protective micronutrients: strontium citrate, vitamins D<sub>3</sub> and K<sub>2</sub> could improve bone health without affecting or even improving health related QOL in postmenopausal osteopenic women. The MOTS is among the first randomized clinical trials to utilize melatonin to prevent bone loss in a postmenopausal cohort by intervening at a critical time during a woman's life, where susceptibility to bone loss is high. This study utilizes a safe, complementary combination therapy MSDK based on the hypothesis of *chronosynergy*—a novel treatment approach using several condition-targeted bone restorative agents with melatonin to reverse bone loss hopefully reducing the need for osteoporosis medications later. With continued study and validation, MSDK could become an early treatment option in the time-course for managing postmenopausal and age-related bone loss; and could potentially play a great role in changing the course of global epidemic of osteoporosis by reducing its rising incidence, tremendous health and medical system burden and high costs of the current status-quo of osteopenia treatment management.

While melatonin therapy alone was protective to bone in a similar cohort (i.e. postmenopausal women with osteopenia) in MelaOst ([Amstrup, Sikjaer et al. 2015](#)) or in healthy perimenopausal cohort in MOPS ([Kotlarczyk, Lassila et al. 2012](#)), there were differences between the MOPS and MelaOst studies and the MOTS. For example, in the MelaOst trial, one-year supplementation with melatonin (1 to 3mg/day) dose-dependently increases femoral neck BMD by 1.4% sites in postmenopausal osteopenic cohort (n=81), but does not affect BMD at other sites ([Amstrup, Sikjaer et al. 2015](#)). In the MOTS, melatonin (5mg) in combination with other three micronutrients: strontium citrate (450mg), vitamin D<sub>3</sub> (2000IU) and vitamin K<sub>2</sub> (60mcg) per day

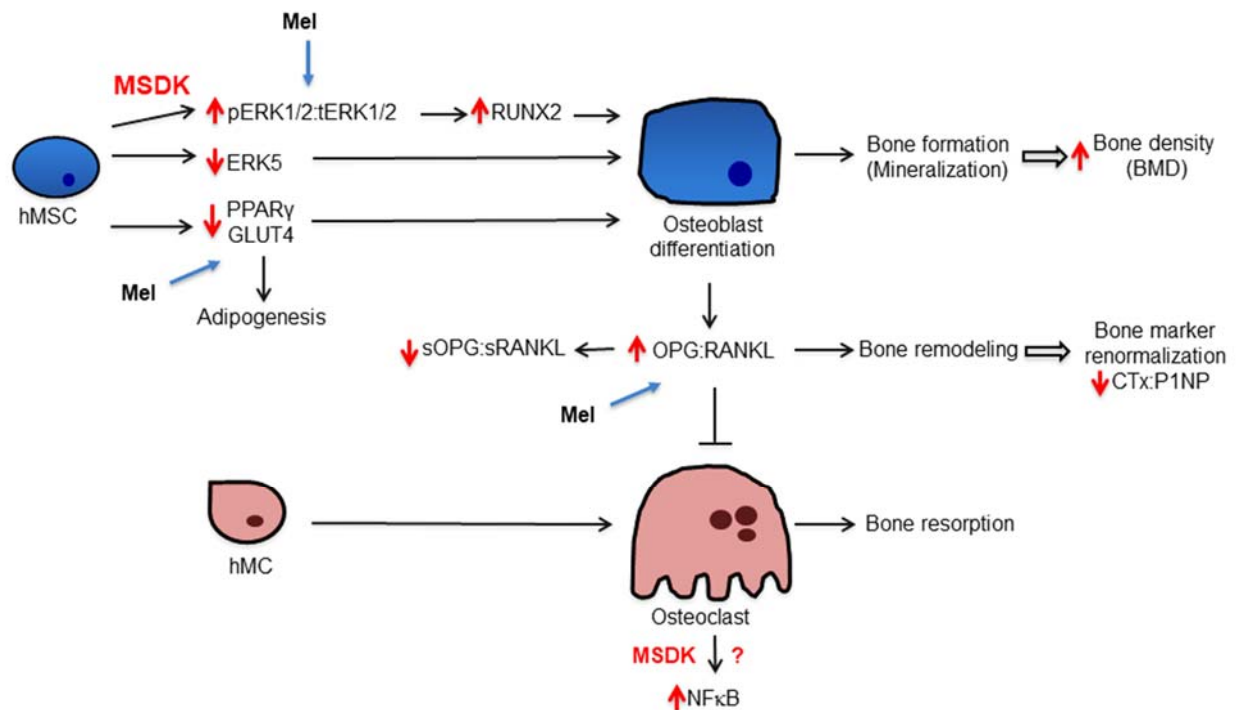
(MSDK) significantly increases lumbar spine BMD by 4.3% and left femoral neck BMD by 2.2%, with a trend ( $p=0.069$ ) towards an increase in hip BMD from baseline after one year in a similar postmenopausal osteopenic cohort ( $n=22$ ). Consequently, the 10-year vertebral fracture risk probability is decreased by 6.48% with MSDK, as compared to 10.8% increase in placebo. MSDK also reduces bone marker turnover ( $\downarrow$ CTx:P1NP ratios) in postmenopausal osteopenic women primarily by increasing the bone formation marker P1NP, and maintaining the bone resorption marker CTx at a steady level. Although melatonin treatment for 6 months renormalizes bone marker turnover ( $\downarrow$ NTx:OC) in the healthy perimenopausal women in the MOPS ([Kotlarczyk, Lassila et al. 2012](#)), no such effects on bone marker turnover occur in postmenopausal osteopenic women in the MelOst ([Amstrup, Sikjaer et al. 2015](#)). Melatonin treatment decreases total fat mass and increases lean body mass by 2.6% compared to placebo in the MelaOst trial ([Amstrup, Sikjaer et al. 2015](#)); this did not occur with MSDK treatment. Although a significant decrease in the variation of weight change over the course of the MOTS occurred in women taking MSDK but not in women taking placebo, suggesting that there was some effect of MSDK on metabolic parameters in a similar cohort as the MelaOst. Quality of life in both the MOPS and MOTS improved; however, what was improved was unique to each trial. For example, melatonin improves QOL in healthy perimenopausal women in the MOPS by improving their physical symptoms of menopause ([Kotlarczyk, Lassila et al. 2012](#)), and by improving mood and reducing sleep interruption ([Maria, Samsonraj et al. 2017](#)). In the MelaOst trial, melatonin improves sleep quality in an insomniac subgroup of participants but is without effect on overall QOL or sleep in this cohort ([Amstrup, Sikjaer et al. 2015](#)). Whereas, in the MOTS, MSDK supplementation exhibited beneficial effects on the QOL in postmenopausal osteopenic women, by apparently lessening the sexual symptoms of menopause (not significant vs. placebo) and by showing

improvements with respect to sleep. All these findings propose that that melatonin may be a better choice for bone loss prevention in healthy middle-aged women transitioning through menopause and MSDK for the early stage bone loss treatment in postmenopausal women.

*In vitro* studies showed that MSDK may be modulating osteoblast and osteoclast function via the release of the paracrine factors, OPG and RANKL, from osteoblasts. MSDK showed an increase in osteoblast differentiation and mineralization in favor of bone formation which was associated with an increase in RUNX2 expression. Increased pERK1/2:tERK1/2 and decreased total ERK5 levels may be contributing to MSDK-mediated osteoblastogenesis; however, future research is warranted to confirm their role by use of inhibitors selective for MEK1/2 and MEK5. Increased osteoblast differentiation by MSDK led to an increase in the ratio OPG:RANKL production in osteoblasts by increasing OPG and decreasing RANKL expression; these changes in OPG and RANKL would result in an increase in osteoblast differentiation and in the inhibition in osteoclast differentiation, Because MSDK treatment did not completely inhibit osteoclastogenesis, we conclude that MSDK is favoring bone remodeling to proceed towards equilibrium by allowing osteoclastogenesis to some extent since balanced bone remodeling is essential to making and maintaining healthy bone.

The *in vitro* part of this translational study also describes novel signaling cascades (**Figure 33**) underlying MSDK's effects on osteoblastogenesis and osteoclastogenesis that include pERK1/2, pERK5, RUNX2, PPAR $\gamma$  and GLUT4. By comparison and noted in **Figure 33**, melatonin shows similar effects on osteoblastogenesis and osteoclastogenesis to MSDK by decreasing RANKL leading to an increase in the ratio of OPG:RANKL, by increasing pERK1/2 and RUNX2, and by decreasing NF $\kappa$ B, PPAR $\gamma$  and GLUT4—all of these effects were dependent upon the type of co-culture ([Maria, Samsonraj et al. 2017](#)). The differences in the clinical outcomes

using melatonin alone (MOPS, MelaOst) versus a combination therapy (MSDK in the MOTS) suggests that their unique mechanistic actions, especially on osteoclasts, may direct use of melatonin for prevention of bone loss in men and women at risk and at the beginning stages of osteopenia (T-scores close to -1) and use of MSDK in later stages of osteopenia (T-scores < -1.5).



**Figure 33. Potential mechanism underlying MSDK effects on bone formation.**

Overall, these *in vitro* studies support our MOTS clinical trial findings demonstrating that MSDK reduces bone turnover rate by increasing P1NP expression, while maintaining steady levels of CTx. MSDK's effects on osteoblastogenesis was consistent with the increase in bone mineral density that was observed in MOTS clinical study. Results from this study underscore the complexity but therapeutically relevant effects of MSDK on bone cell development and activity making MSDK a viable and potential alternative therapy for managing and/or treating osteopenia in postmenopausal women. However, a large-scale, multicenter RCTs testing the efficacy of MSDK to treat osteopenia are warranted to further clarify the effectiveness of MSDK in the overall

osteopenic population.



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# Chapter 7: Appendix

## I. The study information in *ClinicalTrials.gov*

 U.S. National Library of Medicine

**ClinicalTrials.gov** Find Studies ▾ About Studies ▾ Submit Studies ▾ Resources ▾ About Site ▾

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Home > Search Results > Study Record Detail Save this study

Trial record **2 of 7** for: osteopenia postmenopausal | Pittsburgh, Pennsylvania, U.S.

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### Melatonin-Micronutrients for Osteopenia Treatment Study (MOTS)

**⚠** The safety and scientific validity of this study is the responsibility of the study sponsor and investigators. Listing a study does not mean it has been evaluated by the U.S. Federal Government. Read our [disclaimer](#) for details.

ClinicalTrials.gov Identifier: NCT01870115

**Recruitment Status** ⓘ : Completed

**First Posted** ⓘ : June 5, 2013

**Last Update Posted** ⓘ : February 13, 2017

**Sponsor:**  
Duquesne University

**Information provided by (Responsible Party):**  
Duquesne University

Study DetailsTabular ViewNo Results PostedDisclaimerHow to Read a Study Record

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#### Study Description Go to ▾






**Brief Summary:**

The investigators' long-term goal is to employ novel methods to improve bone formation and bone density in women (and men) with osteopenia or osteoporosis while also decreasing signs and symptoms of degenerative joint and disc disease that commonly accompany bone loss as well as improve quality of life (QOL). These conditions generally begin silently as early as the menopause transition and progress to osteopenia and osteoporosis during the post-menopausal years in aging women. The investigators also envision this will be beneficial in aging andropausal men with these conditions. The investigators postulate that melatonin in novel combination with other natural bone-protective agents may act in a "chronosynergy" manner to prevent and correct these perturbations, reducing the risk of bone fractures, and lessening the stiffness and pain associated with bone, joint and cartilage degeneration and improving quality of life (QOL). The objective here, which is the investigators' next step in pursuit of our goal, is to assess the efficacy of an alternative therapy that uses a novel combination of bone-forming agents, melatonin, strontium (citrate)/ vitamin K2 (MK7), and vitamin D3 on bone health in a postmenopausal population. Melatonin is a novel alternative to current treatment(s) because it has multiple bone-protective and sleep-promoting activities within the body, and it is relatively safe so it can be used in an aging population without untoward side effects; strontium and vitamin D3 are shown to enhance bone mineralization and improve post-menopausal osteoporosis. The project goal is to identify if this combination therapy improves bone health and QOL compared to women taking placebo. The investigators' central hypothesis is that combination therapy using melatonin, strontium, vitamin K2, and vitamin D3 will improve bone health and overall QOL in postmenopausal women not taking this regimen by reducing osteoclast activity and increasing osteoblast activity and by improving subjective measures of stress, anxiety, depression and menopause-related symptoms.

| Condition or disease ⓘ | Intervention/treatment ⓘ   | Phase ⓘ |
|------------------------|--|---------|
| Osteoporosis           | Dietary Supplement: Fiber Pill                                       | Phase 1 |
| <b>Osteopenia</b>      | Dietary Supplement: Melatonin, Strontium citrate, Vitamins D3 and K2 |         |

## Study Design

Go to

**Study Type**  : **Interventional (Clinical Trial)**  
**Actual Enrollment**  : 23 participants  
**Allocation**: Randomized  
**Intervention Model**: Parallel Assignment  
**Intervention Model Description**: Melatonin (5mg), strontium (citrate; 450mg) vitamin D3 (2000IU)and vitamin K2 (MK7; 60mcg)  
**Masking**: Quadruple (Participant, Care Provider, Investigator, Outcomes Assessor)  
**Primary Purpose**: Treatment  
**Official Title**: Phase 1 Study of Combination Strontium, Melatonin and Nutritional Co-factors on Bone Health and Quality of Life in **Postmenopausal Women With Osteopenia**  
**Study Start Date**  : August 2013  
**Primary Completion Date**  : February 9, 2017  
**Study Completion Date**  : February 9, 2017

### Resource links provided by the National Library of Medicine



MedlinePlus related topics: [Osteoporosis](#) [Vitamin D](#) [Vitamin K](#)

Drug Information available for: [Melatonin](#)

[U.S. FDA Resources](#)

## Arms and Interventions

Go to

| Arm   | Intervention/treatment    |
|--|--|
| Placebo Comparator: Fiber pill<br>2 plant fiber pills taken p.o. (by mouth) nightly for one year   | Dietary Supplement: Fiber Pill<br>This fiber pill has been manufactured to mimic the pill that contains the dietary supplements melatonin (M), strontium citrate (S), vitamin D3 (D) and vitamin K2 (K) in appearance but does not contain the supplements |
| Active Comparator: strontium/melatonin/Vitamins K2 and D3<br>2 pills taken p.o. (by mouth) nightly for one year. Each pill contains strontium citrate (225 mg), melatonin (2.5 mg), Vitamin K2 (MK7) (30 mcg) and Vitamin D3 (1000 IU) | Dietary Supplement: Melatonin, Strontium citrate, Vitamins D3 and K2<br>Each pill has been manufactured to contain the dietary supplements 2.5mg melatonin (M), 225mg strontium citrate (S), 1000IU vitamin D3 (D) and 30mcg vitamin K2 (K)                |

## Outcome Measures

Go to

### Primary Outcome Measures

1. Changes in bone mineral density from baseline to one year following treatment [ Time Frame: One year ]

Information from the National Library of Medicine



Choosing to participate in a study is an important personal decision. Talk with your doctor and family members or friends about deciding to join a study. To learn more about this study, you or your doctor may contact the study research staff using the contacts provided below. For general information, [Learn About Clinical Studies](#).

Ages Eligible for Study: Child, Adult, Senior  
Sexes Eligible for Study: Female  
Accepts Healthy Volunteers: Yes

Criteria

Inclusion Criteria:

- postmenopausal
- must be osteopenic (T-score between -2.5 and -1)
- willingness to participate in the 12-month study
- willingness to undergo testing of bone turnover markers before and after the drug therapies
- willingness to provide a self-assessment on quality of life throughout the program
- willingness to take their treatments right before bed
- willingness to not to consume alcohol with this medication

Exclusion Criteria:

- women in whom osteopenia is a result of some other known process (e.g. hyperparathyroidism, metastatic bone disease, multiple myeloma or chronic steroid use).
- women on osteoporotic drugs, hypnotics, CYP1A2 inhibiting drugs, fluvoxamine
- women with severe sleep apnea, severe COPD and those with moderate or severe hepatic or renal impairment.

Information from the National Library of Medicine



To learn more about this study, you or your doctor may contact the study research staff using the contact information provided by the sponsor.

Please refer to this study by its ClinicalTrials.gov identifier (NCT number): **NCT01870115**

Locations

United States, Pennsylvania

Duquesne University Center for Pharmacy Care  
Pittsburgh, Pennsylvania, United States, 15282

Sponsors and Collaborators

Duquesne University

Investigators

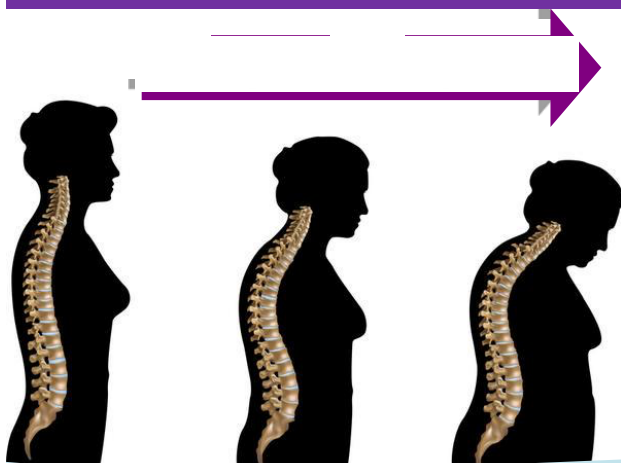
Principal Investigator: Paula A Witt-Enderby, PhD Duquesne University  
Principal Investigator: Mark Swanson, ND Private Practice

## II. Materials used for study advertisements

### A. Flyer

*Did you know that ...*

bones begin to thin during menopause and this silent attack on bones can lead to osteoporosis?



*The early stage of osteoporosis is called **osteopenia***

*If left untreated, **osteopenia** can lead to brittle bone disease called **osteoporosis***

If you are a **postmenopausal woman** concerned about thinning bones, then you may want to participate in a **clinical trial** evaluating the effects of a natural combination therapy on improving bone health

*The study will be conducted at **Duquesne University** by qualified health care professionals*

Apply now

412-396-4296

DUQUESNE UNIVERSITY

Center for Pharmacy Care



Duquesne University  
IRB – Protocol #13-59  
Approval date: May 23, 2013

#### **Participants must-**

- be postmenopausal women
- show evidence of thinning bones (*bone density T-score -1 to -2.5*)
- commit to complete a 12-month study

#### **If eligible, participants will receive-**

- free bone screenings and health assessments
- compensation for each visit and free parking



## B. Newspaper and DU daily articles

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**PG Feed Me** Want updates on restaurant openings, closings, trends and even PG-tested recipes? Get a free newsletter.



# Health notes: Bone-loss study seeks 20 women volunteers

could be a deck if enedjj



PITTSBURGH POST-GAZETTE

MAR 3, 2014

9:32 PM



A Duquesne University research team is seeking participants for a human clinical trial to examine whether a formulation of melatonin, strontium citrate and vitamins D3 and K2 can treat bone loss in women with a thinning-bone condition known as osteopenia.

Paula Witt-Enderby, a Duquesne professor of pharmacology, and Mark Swanson, a naturopathic physician, are seeking 20 female postmenopausal volunteers who've been diagnosed with osteopenia-related bone loss and who are considering treatment to increase bone density.

Each study participant will be asked to keep a diary and complete seven visits.

Participants also will receive two free scans to measure lumbar-spine and hip-bone density; free blood tests for bone formation cells, vitamin D3 and melatonin levels; free clinical health assessments, symptom and quality of life questionnaires; free study medications; and free parking.

Ms. Witt-Enderby conducted a human clinical trial three years ago that revealed that melatonin -- a natural molecule released nightly in the body and by a popular over-the-counter sleep aid -- helped to prevent bone loss in healthy women entering menopause.

Strontium also has been shown to promote healthful effects in most organs and tissues of the body, including bone. The team hypothesizes that strontium and melatonin will have a synergistic effect in bone greater than strontium alone and at a lower dose. Mr. Swanson said strontium's impact on bone formation is much more powerful than calcium.

Women interested in participating should send an email to [marias@duq.edu](mailto:marias@duq.edu).

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### Pro Football

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Article URL: <http://www.post-gazette.com/news/health/2014/03/04/Bone-loss-study-seeks-20-women-volunteers/stories/201403040055>



## Duquesne researchers seeking volunteers for bone loss study



DEBRA ERDLEY | Tuesday, July 30, 2013, 7:23 p.m.

A team of Duquesne University researchers is seeking volunteers for a clinical trial that will attempt to treat bone loss with a combination of melatonin, vitamins and minerals.

A Duquesne spokesman said the trial will build on the success of one Duquesne pharmacology professor Paula Witt-Enderby conducted three years ago. It found that melatonin, a natural mineral released by the body and a popular over-the-counter sleep aid, helped prevent bone loss in healthy women entering menopause.

"Many women are worried about having to take a powerful drug for months to years before they see results, so a more natural treatment is appealing," Witt-Enderby said.

She is seeking 20 postmenopausal volunteers who have been diagnosed with osteopenia-related bone loss for the new trial this month.

EMAIL NEWSLETTERS

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Article URL: <http://triblive.com/news/allegheeny/4448150-74/bone-duquesne-loss>



## Naturopathic Physicians: Natural Medicine. Real Solutions.

Home > AANP Monthly > Bone Loss Study Announced

### Bone Loss Study Announced

Seattle, WA, Aug 6 – Mark Swanson, ND (shown on the right), naturopathic physician from Sequim, Washington (Bastyr, 1984) has been treating his osteopenia / osteoporosis patients with his FOOT Plan (Fully Optimized Osteoporosis Therapy) for the past seven years. The results have consistently shown improvements in bone density and fracture reductions. (NDNR, Jan 2010, www.NDNR.com).



The FOOT Plan's combination of all natural ingredients has eliminated the need for osteoporosis drugs in the majority of his patients. "In our experience it works better, faster and is safer. A unique aspect of the protocol is the concept of melatonin *chronosynergy*, which combines strontium and melatonin together at night to improve the anabolic effects on bone formation", said Swanson.

Dr. Paula Witt-Enderby, professor of pharmacology at Duquesne University, PA has recently published melatonin research on bone loss too. Her MOPS study (Melatonin Osteoporosis Prevention Study) published in the *J Pineal Research*, May 2012, examined whether melatonin, a natural pineal hormone and over-the-counter sleep aid could help prevent bone loss in healthy women entering menopause. The answer was yes.

Now both are collaborating and expanding on each others work with melatonin and bone support to include a follow-up study with women diagnosed with osteopenia. With a translational research grant from Duquesne Mylan School of Pharmacy and the study formulation provided by Pure Encapsulations, Inc., Sudbury, Mass., Witt-Enderby and her research team with Dr. Swanson will study a unique nutraceutical formulation consisting of a combination of melatonin, strontium citrate, with vitamin's D3 and K2. "The population burden of fractures occurs far more with osteopenia than osteoporosis. That's the best time to intervene", said Swanson.

"Melatonin has profound health promoting effects in many organs and tissues of the body, including bone. We hypothesize that melatonin will have a chronosynergy effect with strontium in bone that is greater than strontium alone and at a lower dose. We think it makes better bone building sense when given both together at night," said Witt-Enderby.

The mineral strontium outperforms calcium for increasing bone formation and stopping bone loss. It's safe and starts to increase bone density and reduce fractures within weeks. Vitamin D3 and K2 (MK7) are included in the study formula because they also have a synergy effect on bone strength and quality. "We think melatonin will also act as a catalyst and give an extra bone building boost to the other three ingredients. Besides sleep quality, melatonin is good for the heart, blood sugar, blood pressure and belly fat too. It's icing on the cake", Swanson added.

The clinical trial is scheduled to start in late summer 2013, and will be based in the Duquesne Center for Pharmacy Care, Uptown, PA. Members of Dr. Witt-Enderby's study team will conduct the double blind, placebo controlled study. Osteopenia participants will be assigned to take either two capsules of the study formulation or a placebo at bedtime for one year. Each will have DXA scans (dual-energy X-ray absorptiometry), bone turnover markers tests, health assessments and be asked to keep diaries. A third arm of the study will also test each formulation ingredient on cell culture lines for bone associated responses.

#### Article URL:

[http://www.naturopathic.org/article\\_content.asp?edition=101&section=154&article=826](http://www.naturopathic.org/article_content.asp?edition=101&section=154&article=826)

# Clinical trial to examine natural bone treatment

By KAREN FERRICK-ROMAN

A Duquesne University pharmacology professor is starting a clinical trial to study whether a combination of melatonin, vitamins and minerals successfully treats bone loss.

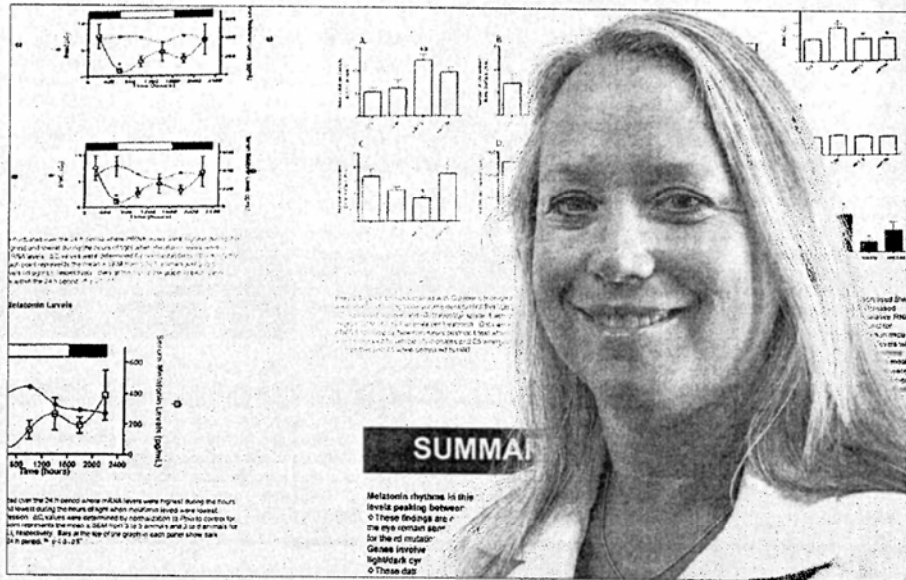
Three years ago, Dr. Paula Witt-Enderby, Duquesne professor of pharmacology, conducted a trial examining whether melatonin, a natural molecule released nightly in the body and a popular over-the-counter sleep aid, could help prevent bone loss in healthy women entering menopause. The answer was yes.

Building on this study, Witt-Enderby's work, now in collaboration with Dr. Mark Swanson, co-investigator and a naturopathic physician, has moved from prevention to treatment. With a Mylan School of Pharmacy translational research grant and a study formulation from Pure Encapsulations Inc. of Sudbury, Mass., Witt-Enderby's team will conduct a clinical trial to examine whether a formulation of melatonin, strontium citrate and vitamins D3 and K2 can treat bone loss in women with thinning bones (osteopenia).

"Strontium has profound health promoting effects in most organs and tissues of the body, including bone. We hypothesize that melatonin will have an enhancing or 'synergistic' effect with strontium in bone that is greater than strontium alone and at a one-third lower dose," Swanson said, explaining that the mineral strontium's impact on bone formation is much more powerful than calcium's. "We think it makes better bone sense when given all together at night to produce a time-dependent enhancement of bone formation or 'chrono-synergy' effect."

"Current drug treatments for osteoporosis are not ideal," Witt-Enderby said. "They have only a 30 percent compliance rate, which really drops after six months. What's needed is a convenient, safer and better-tolerated treatment. Many women are worried about having to take a powerful drug for months to years before they see results, so a more natural treatment is appealing. In the end, it's all about safely preventing fractures and improving quality of life."

The trial will be based in the Center for Pharmacy Care located in the Muldoon Building at 1000 Fifth Ave. For the double-blind, placebo-controlled study, Witt-Enderby seeks 20 female post-



**Dr. Paula Witt-Enderby of Duquesne University, who has previously studied the effects of melatonin, will conduct a clinical trial examining treatment of bone loss.**

menopausal volunteers who have been diagnosed with osteopenia-related bone loss and who are considering treatment to increase bone density. Each study participant will be asked to keep a diary and complete seven visits.

- Participants in the yearlong trial will receive:
- Two free DXA scans to measure lumbar spine and hip bone density
  - Free blood tests for bone formation cells, vitamin D3 and melatonin levels
  - Free clinical health assessments, symptom and quality of life questionnaires
  - Free study medications
  - Free parking.

Members of Witt-Enderby's team include graduate assistant Sifat Maria, and Duquesne professors Drs. Holly Lasilla and Chris O'Neil. For more information about participating contact Maria at 412-396-4296 or marias@du.edu

Ferrick-Roman is media relations manager at Duquesne University.



**Harsha Rathi, far left, a student in the masters of science in Biotechnology program at Duquesne, and Sifat Maria, a doctoral student in the university's Graduate School of Pharmaceutical Sciences, work with Witt-Enderby.**

Article published at Senior Living of *Pittsburgh Catholic* on Friday, August 16, 2011



News

News Coverage for Duquesne

Office of Marketing and Communications  
4th Floor Koren Building  
718 Fifth Avenue  
Pittsburgh, PA 15219  
Phone: 412.396.6050  
Fax: 412.396.2243

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## Clinical Trial at Duquesne University to Examine Natural Bone Treatment

July 30, 2013

In August, a Duquesne University pharmacology professor will start a clinical trial to study whether a combination of melatonin, vitamins and minerals successfully treats bone loss.

Three years ago, Dr. Paula Witt-Enderby, Duquesne professor of pharmacology, conducted a trial examining whether melatonin, a natural molecule released nightly in the body and a popular over-the-counter sleep aid, could help prevent bone loss in healthy women entering menopause. The answer was yes.

Building on this study, Witt-Enderby's work, now in collaboration with Dr. Mark Swanson, co-investigator and a naturopathic physician, has moved from prevention to treatment. With a Mylan School of Pharmacy translational research grant and a study formulation from Pure Encapsulations Inc. of Sudbury, Mass., Witt-Enderby's team will conduct a clinical trial to examine whether a formulation of melatonin, strontium citrate and vitamins D3 and K2 can treat bone loss in women with thinning bones (osteopenia).

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The trial will be based in the Center for Pharmacy Care located in the Muldoon Building at 1000 Fifth Ave. For the double-blind, placebo-controlled study, Witt-Enderby seeks 20 female postmenopausal volunteers who have been diagnosed with osteopenia-related bone loss and who are considering treatment to increase bone density. Each study participant will be asked to keep a diary and complete seven visits.

Participants in the yearlong trial will receive:

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- Free parking.

Members of Witt-Enderby's team include graduate assistant Sifat Maria, and Duquesne professors Drs. Holly Lasilla and Chris O'Neil. For more information about participating, contact Maria.

### Duquesne University

Founded in 1878, Duquesne is consistently ranked among the nation's top Catholic universities for its award-winning faculty and tradition of academic excellence. Duquesne, a campus of nearly 9,500 graduate and undergraduate students, has been nationally recognized for its academic programs, community service and commitment to sustainability. Follow Duquesne University on [Facebook](#), [Twitter](#) and [Instagram](#).  
[www.duq.edu](http://www.duq.edu)

Article URL: <http://www.duq.edu/news/clinical-trial-at-duquesne-university-to-examine-natural-bone-treatment>

### III. Initial visit form

INITIAL VISIT FORM

ID \_\_\_\_\_

NAME (First, Last): \_\_\_\_\_

Date: \_\_\_\_\_

**VISIT CHECKLIST**

- Explain and obtain signed one copy of **Study consent form** & give another Initials: \_\_\_\_\_
- Explain compensation; obtain completed **Payment form** Initials: \_\_\_\_\_
- Explain and obtain signed **BP and Blood consent form** Initials: \_\_\_\_\_
- Baseline Intake & FRAX** Assessment forms- filled Initials: \_\_\_\_\_
- Blood pressure** taken (Total of 3 times, record below) Initials: \_\_\_\_\_  
*Pressure lower than 100/60 or higher than 140/90 → NOT ELIGBLE*
- Achilles measurement** taken (record below) Initials: \_\_\_\_\_  
*T-score -2.5 or lower → NOT ELIGBLE, refer to physician for follow-up*
- Height and weight** taken (attached with FRAX form) Initials: \_\_\_\_\_
- Blood sample** taken (Time: \_\_\_\_\_ AM/ PM) Initials: \_\_\_\_\_
- Quality of Life questionnaires** (MENQOL, PSS, STAI, CES-D) given Initials: \_\_\_\_\_
- DXA scan report** (if **NOT have**, instruction given and explained) Initials: \_\_\_\_\_
- Diary, Instruction, Medication (2bottles) and Gift** given Initials: \_\_\_\_\_
- Schedule next visit; appointment card given Initials: \_\_\_\_\_  
 Next visit – Date: \_\_\_\_\_ Time: \_\_\_\_\_ AM / PM
- Parking sticker given (if necessary) Initials: \_\_\_\_\_

**FOR OFFICE USE ONLY**

|                                  |                     |                                     |
|----------------------------------|---------------------|-------------------------------------|
| Achilles Measurement _____       | DXA score _____     | Initials: _____                     |
| FRAX measurement _____           |                     | Initials: _____                     |
| Blood Pressure: 1) _____ / _____ | 2) _____ / _____    | 3) _____ / _____ Avg. _____ / _____ |
| Melatonin _____                  | Date Entered: _____ | Initials: _____                     |
| Vitamin D3 _____                 | Date Entered: _____ | Initials: _____                     |
| Osteocalcin _____                | Date Entered: _____ | Initials: _____                     |
| NTX _____                        | Date Entered: _____ | Initials: _____                     |

#### IV. Study medication label

|  |             |
|--|-------------|
| <b>Melatonin-micronutrients for Osteopenia Treatment Study (MOTS)</b>  |             |
| Duquesne University-School of Pharmacy<br>600 Forbes Ave, Pittsburgh PA 15282  | Date: _____ |
| <b>Patient :</b> _____   |             |
| Qty: 60 capsules   |             |
| <b>Investigational Protocol: melatonin 2.5 mg / strontium (citrate) 225 mg / K2 30 mcg / D3 1000 IU or Placebo (lactose)</b> |             |
| Take two capsules daily before bed with a full glass of water.   |             |
| Primary Investigator: Paula A. Witt-Enderby, Ph.D.<br>Nurse On-call: 412-580-1952  |             |

## V. Bone density T-scores at baseline and at the end of the treatment

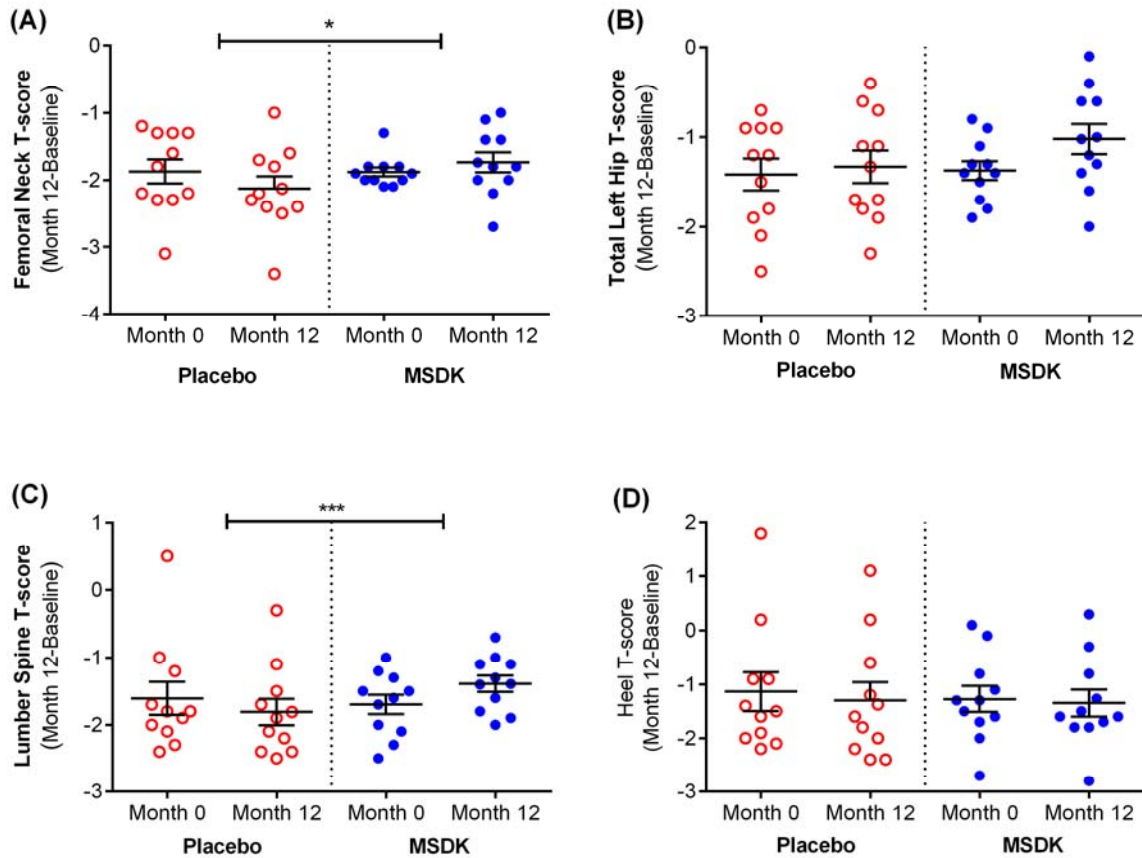
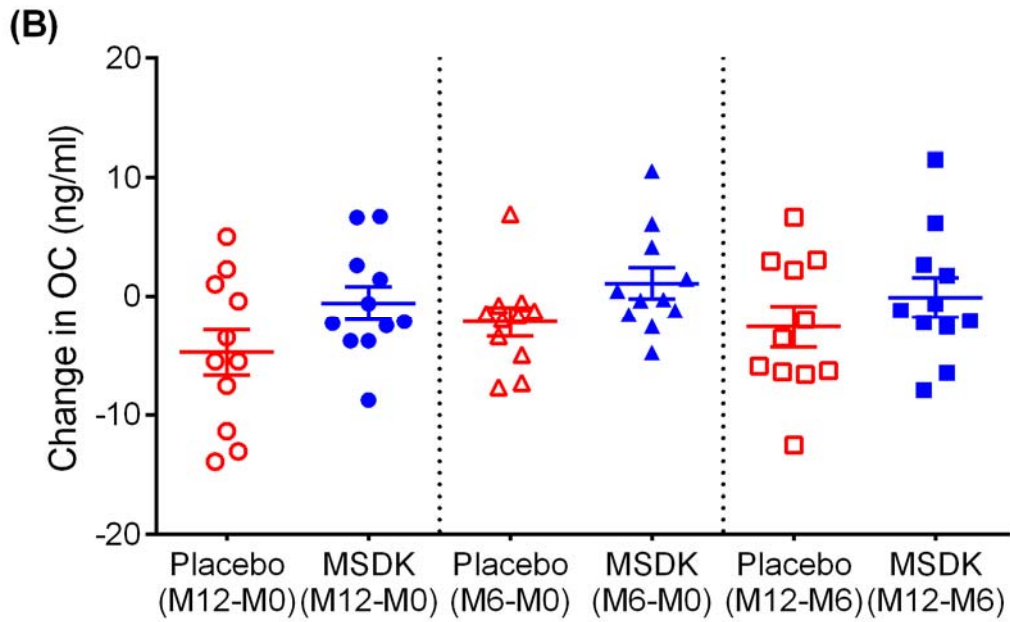
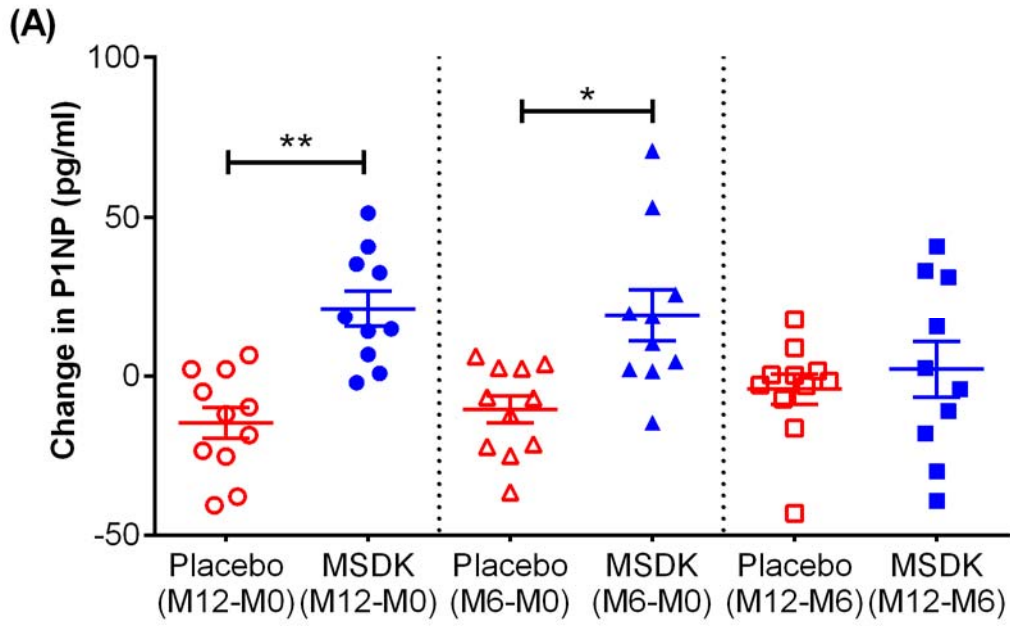


Figure V. Bone mineral density in the left femoral neck, total left hip and lumbar spine area was measured via DXA and Achilles (n=11 per group). Each dot in scatter plot represents the bone mineral density t-score of individual participant at baseline and at month 12, in the (A) left femoral neck, (B) total left hip, (C) lumbar spine (L1-L4) and (D) heel area, respectively for placebo (open bar) and MSDK (closed bars). \* $p \leq 0.05$  and \*\*\* $p \leq 0.001$  vs. placebo for changes in T-scores from baseline to month 12.

## VI. Treatment effects on bone marker changes in every six months



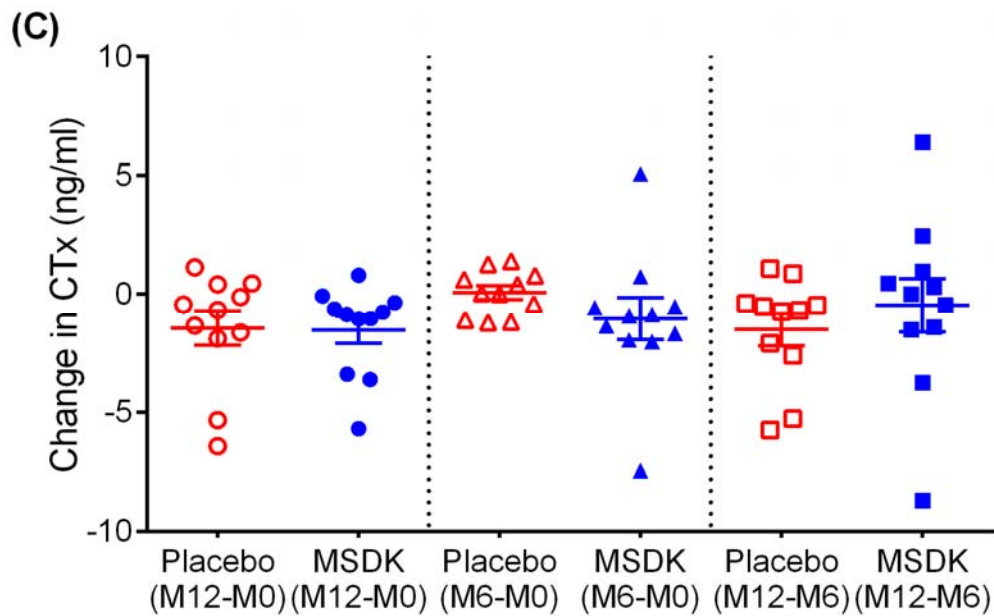


Figure VI. Bone formation marker, (A) total procollagen type 1 amino-terminal propeptide (P1NP) and (B) osteocalcin (OC; both intact and N-terminal mid-fragments); and (C) bone resorption marker Collagen Type I C-Telopeptide (CTx) were measured at months 0, 6, and 12, respectively via ELISA (n=11 per group). Each dot in scatter plot represents the mean ( $\pm$  S.E.M.) change in bone marker of a participant from baseline to month 12 and then in every six months (baseline to month 6, month 6 to month 12), respectively for placebo (open dots) and MSDK (closed dots). \* $p \leq 0.05$  and \*\* $p \leq 0.01$  vs. placebo, one-way ANOVA followed by Bonferroni post hoc  $t$ -test.

## VII. Bone markers (P1NP, OC and CTx) of participants enrolled in fall and spring

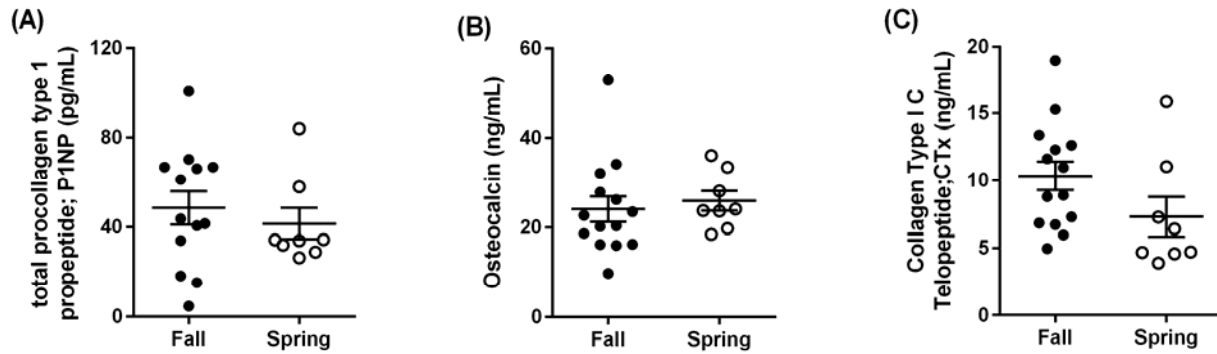


Figure VII. Bone markers, **(A)** total procollagen type 1 amino-terminal propeptide (P1NP) and **(B)** osteocalcin (OC; both intact and N-terminal mid-fragments); and **(C)** bone resorption marker Collagen Type I C-Telopeptide (CTx) of participants enrolled in fall (n=14, closed dots) and spring (n=8, open dots), respectively. \* $p \leq 0.05$  vs. placebo, unpaired two tailed  $t$ -test with Welch's correction.

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