Daily Nocturnal Melatonin Supplementation in Perimenopausal Women: A Randomized, Double-Blind, Placebo-Controlled Pilot Study Examining the Effects of Melatonin on Bone Health and Quality of Life

Mary Kotlarczyk

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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
Mary P. Kotlarczyk

December 2011
DAILY NOCTURNAL MELATONIN SUPPLEMENTATION IN PERIMENOPAUSAL WOMEN: A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED PILOT STUDY EXAMINING THE EFFECTS OF MELATONIN ON BONE HEALTH AND QUALITY OF LIFE

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Mary P. Kotlarczyk

Approved July 14, 2011
ABSTRACT

DAILY NOCTURNAL MELATONIN SUPPLEMENTATION IN PERIMENOPAUSAL WOMEN: A RANDOMIZED, DOUBLE-BLIND, PLACEBO-CONTROLLED PILOT STUDY EXAMINING THE EFFECTS OF MELATONIN ON BONE HEALTH AND QUALITY OF LIFE

By
Mary P. Kotlarczyk

December 2011

Dissertation supervised by Paula A. Witt-Enderby, Ph.D.

Objective: The purpose of this pilot study was to assess the effects of nightly melatonin supplementation on primary endpoints of bone health and secondary endpoints of quality of life in perimenopausal women.

Methods: A total of 18 perimenopausal women were randomized to receive melatonin, 3 mg p.o., (n=13) or placebo (n=5) nightly for six months. Bone density was measured by calcaneal ultrasound. Osteocalcin (OC), a bone formation marker, and amino-terminal cross-linking telopeptide of type I collagen (NTX), a bone resorption marker, were measured in serum samples taken bimonthly to assess changes in bone turnover. Participants completed the Menopause-Specific Quality of Life-Intervention (MENQOL) and Pittsburgh Sleep Quality Index (PSQI) questionnaires before treatment and after six months. Subjects kept daily diaries recording menstrual cycling, well-being, and sleep patterns.
**Results:** No significant differences in bone density or bone turnover markers were found with melatonin supplementation; however, there was a time-dependent trend in the melatonin group towards an NTX:OC ratio of 1:1. A ratio of 1:1 could be an indication of the ability of melatonin to balance bone resorption and bone formation to maintain bone mass. Melatonin had no effect on vasomotor, psychosocial, or sexual MENQOL domain scores after six months of nocturnal supplementation. However, women taking melatonin had significant improvement in physical MENQOL domain scores compared to placebo (-0.6 ± 0.8 and 0.1 ± 0.5, respectively). There were no significant changes in PSQI score or average number of hours slept with melatonin treatment. Analysis of menstrual cycling data showed women in the melatonin group had significantly fewer periods (4.3 ± 0.6, n = 10 melatonin; 6.5 ± 0.3, n = 4 placebo; mean ± SEM) and longer days between periods (51.2 ± 11.4, n = 10 melatonin; 24.1 ± 0.9, n = 4 placebo) but showed no difference in duration of bleeding. Melatonin supplementation was well-tolerated.

**Conclusions:** Melatonin may have the ability to regulate serum markers of bone metabolism, indicating a potential role for melatonin in restoring imbalances in bone remodeling and prevention of bone loss. The improvement in quality of life with melatonin indicates supplementation may be beneficial for women experiencing physical symptoms of menopause. Further investigation into how melatonin impacts bone health and quality of life is warranted.
DEDICATION

To all my family and friends who helped make this possible, thank you for your never-ending support. Without your love and encouragement, I would not have made it this far. This dissertation is as much yours as it is mine. Thank you.
ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

AANAT – arylalkylamine N-acetyltransferase
AIS – adolescent idiopathic scoliosis
BAP – bone-specific alkaline phosphatase
BCE – bone collagen equivalents
BMD – bone mineral density
BTM – bone turnover marker
cAMP – cyclic adenosine monophosphate
CTX – carboxy-terminal cross-linking telopeptide of type I collagen
DPD – deoxypyridinoline
DXA – dual-energy x-ray absorptiometry
ERK 1/2 – extracellular signal-regulated kinase 1/2
FSH – follicle-stimulating hormone
GnRH – gonadotropin-releasing hormone
hAMSC – human adult mesenchymal stem cell
LH – luteinizing hormone
MAPK – mitogen-activated protein kinase
MEK 1/2 – mitogenic extracellular kinase 1/2
MENQOL – Menopause-specific Quality of Life questionnaire
MT₁ – Melatonin receptor 1
MT₂ – Melatonin receptor 2
NTX – amino-terminal cross-linking telopeptide of type I collagen
OC – osteocalcin
OPG – osteoprotegerin
PICP – procollagen type I C propeptide
PINP – procollagen type I N propeptide
PSQI – Pittsburgh Sleep Quality Index
PTH – parathyroid hormone
PYD – pyridinoline
RANKL – receptor activator of NF-κB ligand
SCN – suprachiasmatic nucleus
SERM – selective estrogen receptor modulator
STRAW – Stages of Reproductive Aging Workshop
SWAN – Study of Women's Health Across the Nation
TRAP – tartrate-resistant acid phosphatase
INTRODUCTION

Perimenopause

A women’s reproductive health changes throughout her lifetime. The typical female reproductive cycle consists of three phases: the follicular phase, ovulation, and the luteal phase. During the follicular phase, oocyte-containing follicles develop in the ovary and secrete estradiol. Estradiol acts at the hypothalamus to trigger release of gonadotropin-releasing hormone (GnRH), which activates receptors on the anterior pituitary gland. Activation of these receptors induces secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH stimulates ovarian follicles to develop further, with one dominant follicle becoming destined for ovulation. The follicles continue to release estradiol. Low levels of estradiol inhibit secretion of LH from the pituitary. When estradiol reaches a threshold level, however, it stimulates the release of LH. The resulting surge in LH immediately precedes the release of the mature egg from the follicle. During ovulation, the follicle ruptures and releases the egg from the ovary. The remainder of the follicle develops into the corpus luteum, which secretes estradiol and progesterone. During the luteal phase, the rising progesterone acts in the uterus to thicken the endometrial lining for implantation. Estradiol and progesterone exert negative feedback on the anterior pituitary to lower levels of FSH and LH. Towards the end of the luteal phase, the corpus luteum atrophies and estradiol and progesterone levels diminish. The loss of negative feedback on FSH secretion results in rising levels of FSH. If no fertilization occurs, the endometrial lining is shed during menstruation, and the cycle begins again.¹ As women age, there is a decline in ovarian
function due to depletion of follicle stores. An increasing amount of cycles are anovulatory in which no egg is released. The changes in ovarian function result in irregular patterns of endocrine hormone secretion. These variable changes continue until there is complete cessation of the menstrual cycle (menopause).

The transition through menopause is unique to each woman; however, the majority of women experience a change from regular menstrual cycling to irregular menstrual periods and finally to cessation of menstruation. According to the Stages of Reproductive Aging Workshop (STRAW), the main reproductive stages are classified into three phases: reproductive, menopausal transition, and postmenopause. Stages are described in terms of menstrual cycling and characteristic endocrine changes.

<table>
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<th>Stages:</th>
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<th>-4</th>
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<td>Menopausal Transition</td>
<td>Postmenopause</td>
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<tr>
<td></td>
<td>Early</td>
<td>Peak</td>
<td>Late</td>
<td>Early</td>
<td>Late*</td>
<td>Early*</td>
<td>Late</td>
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<tr>
<td></td>
<td>variable</td>
<td>variable</td>
<td>variable</td>
<td>a 1 yr</td>
<td>b 4 yrs</td>
<td>until demise</td>
<td></td>
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<tr>
<td>Duration of Stage:</td>
<td>variable to regular</td>
<td>regular</td>
<td>variable cycle length (&gt;7 days different from normal)</td>
<td>≥2 skipped cycles and an interval of amenorrhea (≥60 days)</td>
<td>none</td>
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<tr>
<td>Menstrual Cycles:</td>
<td>normal FSH</td>
<td>↑ FSH</td>
<td>↑ FSH</td>
<td>↑ FSH</td>
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<td>Endocrine:</td>
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*Stages most likely to be characterized by vasomotor symptoms↑ = elevated

**Figure 1: Stages of Reproductive Aging.** The diagram above illustrates the main phases in female reproductive aging according to the STRAW meeting including the reproductive stage characterized by normal menstrual cycling, the menopausal transition (perimenopause) stage identified by irregular cycling, and the postmenopause stage when there is cessation of the menstrual cycle. Figure reprinted from Soules MR, Sherman S, Parrott E, et al. Stages of Reproductive Aging Workshop (STRAW). *J Womens Health Gend Based Med.* Nov 2001;10(9):843-848, Copyright 2001, with permission from Mary Ann Liebert, Inc. publishers.
As illustrated in Figure 1, the reproductive phase encompasses the time from menarche to the onset of irregular menstrual cycles, which usually occurs when a women is in her middle to late forties. The menopausal transition phase, including the perimenopause stage, is characterized by variable menstrual cycling length and intervals of amenorrhea (absence of a menstrual period). Perimenopause is defined as the stage immediately before menopause and extending to the first year after menopause. The postmenopause phase is the year after the final menstrual period to the end of life.

As noted, there is some overlap in the definition of stages, particularly concerning the year after the final menstrual period. There is also ambiguity as to the definitive starting and ending points to each stage. In this study, we chose to study melatonin supplementation in perimenopausal women. There is no clinical test that indicates the start or end of the perimenopausal state. An extensive review of the literature concluded that perimenopause is characterized not by declining estrogen levels as traditionally accepted; instead, perimenopausal women have periods of high estrogen compared to premenopausal women.\textsuperscript{4} Estrogen and FSH levels fluctuate and are typically higher in perimenopausal women.\textsuperscript{5} Due to the frequent fluctuations in reproductive hormones, perimenopause is often accompanied by symptoms such as difficulty sleeping, vaginal dryness, and vasomotor symptoms like hot flashes.\textsuperscript{6, 7} Signs of bone loss also begin to occur during perimenopause, indicating the deterioration of bone seen in postmenopausal osteoporosis may begin earlier than the onset of menopause.\textsuperscript{8-10}

**Osteoporosis**

Osteoporosis is a bone disease that develops when there is an imbalance in bone remodeling. Bone is constantly changing, undergoing a process of breakdown and
rebuilding. The remodeling process is under the control of two types of bone cells:
osteoclasts and osteoblasts. Osteoclasts are responsible for the resorption of old bone,
creating cavities, which are then replaced with new bone matrix by osteoblasts (Figure 2).

![Normal Bone Remodeling](image.png)

**Figure 2: The bone remodeling process.** Osteoclasts break down bone in the process of
resorption, creating cavities. Bone-forming osteoblasts fill in the areas of bone loss to complete
the remodeling process. Figure reprinted from Doheny MO, Sedlak CA, Estok P, Poirier V. DXA
with permission from Wolters Kluwer Health, Inc.

An imbalance in the activity of osteoclasts and osteoblasts can lead to the development of
osteoporosis.\(^\text{11}\) It is believed that in osteoporosis, osteoclast activity outpaces osteoblast
activity, such that more bone is broken down than is being formed. This imbalance in
bone remodeling leads to an overall loss of bone mass and an increased risk of fracture.
In 2005, fractures resulted in $17 billion of direct medical costs. Costs are estimated to
exceed $20 billion by 2025; therefore, research into osteoporosis prevention is crucial.\(^\text{12}\)

**Role of reproductive hormones in osteoporosis**

Women in particular are at greater risk for osteoporosis, accounting for 80% of
Americans diagnosed with osteoporosis.\(^\text{13}\) The increased incidence of osteoporosis in
women has largely been attributed to declining levels of estrogen during the menopausal
transition as decreases in circulating estrogen coincide with rapid bone loss and estrogen replacement therapy is protective against bone loss. Emerging research has shown the importance of other reproductive hormones, which may also play a role in the development of postmenopausal osteoporosis, namely FSH and progesterone.

FSH levels are high during perimenopause, a time of significant bone loss in women. Results from the Study of Women’s Health Across the Nation (SWAN) demonstrate a negative correlation between FSH levels and markers of bone metabolism in premenopausal and early perimenopausal women. A study by Sun et al. showed transgenic mice with reduced FSH levels but with functional reproductive systems had an increase in trabecular bone mass and a decrease in tartrate-resistant acid phosphatase, a serum marker of bone resorption. In vitro experiments showed functional FSH receptors on human osteoclasts but not osteoblasts. The authors postulate that high levels of FSH may increase bone resorption through activation of FSH receptors on osteoclasts. The direct influence of FSH on bone metabolism is of some debate, especially given the results of a recent study in which suppression of FSH in postmenopausal women did not reduce markers of bone resorption. To remove any confounding influences of estrogen on bone, women in both the control and treatment group were given an aromatase inhibitor to decrease estrogen levels. Contrary to the study by Sun et al., reducing FSH levels did not reduce levels on bone resorption markers or increase markers of bone formation. Nevertheless, there is evidence to support a relationship between FSH and bone metabolism either through direct activation of FSH receptors on osteoclasts to stimulate bone resorption or via the modulatory effects of FSH on hormones such as estrogen and progesterone.
A recent review by Seifert-Klaus and Prior details the evidence supporting a role for progesterone in bone health. In fact, several in vitro studies show treatment with progesterone can increase proliferation of human bone cells, with proliferative effects being enhanced with co-administered of estradiol. The fluctuation in estrogen, FSH, and progesterone levels occurring during perimenopause may contribute to the deterioration of bone mass. Research demonstrating the possible roles of FSH and progesterone in bone health indicate the need to examine not only estrogen but other reproductive hormones when considering the pathology of postmenopausal osteoporosis.

**Current Osteoporosis Treatments**

To prevent the bone loss associated with menopause, it is recommended that women have adequate daily intake of calcium and vitamin D. Even with calcium and vitamin D supplementation, women are often prescribed medication to curb bone loss associated with menopause. Most prescription therapies are targeted at inhibiting bone resorption. These include agents such as calcitonin, bisphosphonates, selective estrogen receptor modulators (SERMs), and more recently the receptor activator of NF-κB ligand (RANKL) inhibitor denosumab. The parathyroid hormone (PTH) analog teriparatide is the only FDA approved osteoporosis therapy that increases formation of new bone.

Calcitonin is an endogenous hormone that acts directly on receptors on osteoclasts to decrease bone resorption. Calcitonin is approved for the treatment of postmenopausal osteoporosis 5 years after menopause; however, the development of newer drugs has led to a decline in calcitonin usage. Bisphosphonates are the most widely prescribed therapy for prevention and treatment of postmenopausal osteoporosis. Bisphosphonates are attracted to areas of high bone remodeling based on their ability to associate tightly
with hydroxyapatite, a main mineral component of bone. During bone remodeling, osteoclasts take up bisphosphonate molecules on the bone surface. Bisphosphonates induce apoptosis of osteoclasts to reduce bone resorption. While effective at preventing bone loss and reducing fracture risk, bisphosphonate usage is not without side effects. Women taking bisphosphonates have increased incidence of osteonecrosis of the jaw. Oversuppression of bone remodeling may also result in an increase in microdamage to bone architecture. Concern has risen over a possible increase in atypical femur fracture with long-term bisphosphonate therapy, although there is conflicting data regarding the exact role of bisphosphonates in the occurrence of these fractures. Although not a clinically established guideline, many have suggested bisphosphonate usage be limited to a fixed length of time or that patients be given a “drug holiday” to reduce the risk of untoward effects.

Estrogen replacement therapy can protect against the bone loss associated with menopause; however, there are health concerns with long-term hormone replacement therapy including increased risk of breast cancer and adverse cardiovascular events. The selective estrogen receptor modulator (SERM) raloxifene has been developed and approved in the United States for both the prevention and treatment of postmenopausal osteoporosis. SERMs possess both agonist and antagonist activity with responses being tissue-specific. Raloxifene binds to the estrogen receptor to modulate estrogen-regulated gene transcription. Depending on the tissue of action and the comodulators present, raloxifene can act as an estrogen receptor agonist or antagonist. Raloxifene acts as an estrogen receptor agonist in the bone, resulting in inhibition of osteoclast activity and reduction of bone resorption. While SERMs maintain the beneficial effects of estrogen
on bone, unwanted side effects with therapy include hot flashes, vaginal dryness, and increased risk of venous thromboembolism.\textsuperscript{28} Side effects such as these can affect patient compliance and may limit the duration of treatment.

Denosumab is a new therapy approved in 2010 for the prevention and treatment of postmenopausal osteoporosis. Like bisphosphonates, denosumab decreases bone resorption but does not do so through the same mechanism of inducing osteoclast apoptosis. Denosumab is a monoclonal antibody, which binds to RANKL, an osteoclast activator, thereby reducing osteoclast activity and bone resorption. Twice yearly injections of denosumab for three years significantly reduced the risk of fracture in osteoporotic women.\textsuperscript{29} As this is a new therapy, effects of long-term usage are not yet known.

The parathyroid hormone (PTH) analog teriparatide is effective at increasing bone mass by stimulating osteoblasts to form new bone. Teriparatide is a peptide composed of PTH amino acids 1-34. Parathyroid hormone, like calcitonin, is an endogenous hormone, which works to maintain calcium homeostasis. The action of PTH on bone depends on the dose and length of exposure. Elevated PTH levels over extended periods of time lead to activation of osteoclasts and an overall increase in bone resorption. However, intermittent spikes in PTH levels, such as with once or twice daily injections of teriparatide, stimulate PTH receptors on osteoblasts to increase activity of osteoblasts and increase bone mass.\textsuperscript{30} This anabolic therapy is successful in increasing bone density, improving the microarchitecture of the bone, and reducing risk of vertebral and nonvertebral fractures.\textsuperscript{31} While PTH therapy is effective, patient compliance can be affected by the need for subcutaneous injection. PTH therapy is limited to two-year
duration, at which time antiresorptive therapy is often necessary to maintain bone mass developed while on PTH.

**Bone Turnover Markers**

Bone turnover markers (BTMs) are biochemical indicators of bone remodeling that are typically measured in blood or urine samples. Changes in bone turnover markers occur more rapidly than alterations in bone mineral density (BMD) as measured by dual-energy x-ray absorptiometry (DXA). For this reason, biochemical markers of bone turnover are gaining favor in clinical research, particularly for monitoring patient response to osteoporosis therapies. Markers of bone formation, indicative of osteoblast activity, include: osteocalcin (OC), bone-specific alkaline phosphatase (BAP), procollagen type I N propeptide (PINP), and procollagen type I C propeptide (PICP). With the exception of BAP, all of these markers are products synthesized by osteoblasts. Bone-specific alkaline phosphatase is a membrane-bound enzyme on the osteoblast cell surface. Bone resorption markers reflective of osteoclast activity include: amino-terminal cross-linking telopeptide of type I collagen (NTX), carboxy-terminal cross-linking telopeptide of type I collagen (CTX), deoxypyridinoline (DPD), pyridinoline (PYD), and tartrate-resistant acid phosphatase (TRAP). NTX, CTX, DPD, and PYD are products of type I collagen degradation which are released during bone resorption by osteoclasts. TRAP is an enzyme of osteoclast origin.

Markers of bone turnover can be useful in monitoring responses to therapy. Bone turnover markers typically show a decrease within 3-6 months of starting antiresorptive therapy with bisphosphonates. Anabolic therapy with teriparatide also induces rapid changes in bone turnover markers. The pattern and timing of how bone markers change
with treatment depend on the main mechanism of drug action. For example, bisphosphonates strongly inhibit osteoclast activity. Bone resorption markers decline early in the treatment period (around three months) while bone formation markers do not decrease until approximately four weeks later when there is suppression of overall bone turnover. On the other hand, therapy with the anabolic agent teriparatide significantly increases bone formation markers within three months, reflecting the enhancement of osteoblast activity. Bone resorption markers do not rise significantly above baseline until approximately six months after beginning teriparatide treatment. Nevertheless, bone markers allow for the assessment of treatment effects within a short period of time and reflect the rapid cellular effects of therapy on bone metabolism. Monitoring fluctuations in bone turnover markers can provide more rapid indications of patient compliance with medication and the level of response to therapy. In this study, we utilize OC and NTX to monitor changes in bone turnover markers after treatment with melatonin. These markers were chosen because they have been utilized in previous studies of bone turnover markers of perimenopausal women and are known to be responsive to treatment in postmenopausal women. In addition, these markers can withstand storage before detection which was required due to our rolling enrollment period. Other markers, such as BAP, require more immediate measurement making assessment less conducive for our study.

**Melatonin**

The current study examined melatonin supplementation in perimenopausal women. Melatonin is an endogenous hormone produced in the pineal gland. Melatonin levels are on a circadian cycle with higher levels found during periods of darkness.
Melatonin is synthesized from serotonin in the pineal gland. Serotonin is acetylated by arylalkylamine N-acetyltransferase (AANAT) to yield N-acetylserotonin. Hydroxyindol-O-methyltransferase acts on N-acetylserotonin to yield melatonin. Synthesis of melatonin is under regulation of multisynaptic pathway originating in the suprachiasmatic nucleus (SCN) of the hypothalamus and projecting into the pineal gland. Sympathetic signaling through norepinephrine activates α1 and β1-adrenergic receptors on pinealocytes.

Activation of these receptors results in phosphorylation of transcription factors that regulate production of AANAT, the rate-limiting enzyme in the melatonin synthetic pathway. AANAT levels are highest during the dark, driving production of more melatonin during this period. Light acts to depolarize retinal ganglion cells that innervate the SCN, blocking sympathetic output to the pineal gland, thereby decreasing synthesis of melatonin. Melatonin levels typically begin to rise at dusk and peak around 02:00 hours. Light exposure at night inhibits the nocturnal increase in plasma melatonin and can affect the circadian cycle of melatonin release. A study comparing salivary melatonin secretion in subjects aged 18-45 found a decrease of 0.36 hours in the duration of melatonin secretion for every 10 years of age. Peak levels of melatonin are lower in middle-age and older subjects, with levels being as low as one-eighth that of young adult levels.

Melatonin can exert effects through two known mammalian melatonin receptors: MT1 and MT2. Both receptors are G-protein coupled receptors and signal largely through Gi proteins to decrease cyclic adenosine monophosphate (cAMP) levels. However, signaling pathways initiated by melatonin receptor activation vary depending on the tissue of interest. (reviewed by Dubocovich, et al.) MT1 and MT2 can also act via Gq
proteins to increase intracellular calcium. MT$_2$ has also been shown to decrease cyclic
cyclic guanosine monophosphate (cGMP). Internalization of MT$_2$ receptors on human
adult mesenchymal stem cells (hAMSCs) may play a role in the differentiation process.
It has been proposed that desensitized MT$_2$ receptors in hAMSCs can bind to β-arrestin
proteins creating a scaffold which can interact with proteins in the mitogen-activated
protein kinase (MAPK) pathway including mitogenic extracellular kinase 1/2 (MEK 1/2)
and extracellular signal-regulated kinase 1/2 (ERK 1/2) to induce differentiation.$^{46}$ Aside
from interaction with its receptors, melatonin can exert various effects through its potent
antioxidant and free radical scavenging capabilities.$^{47-49}$

**Melatonin Effects on Bone**

The relationship between melatonin and bone is well-documented.$^{50-52}$ Melatonin
and enzymes essential for melatonin synthesis have been found in both mouse and human
bone marrow cells.$^{53, 54}$ Melatonin levels in bone marrow can be orders of magnitude
higher than those in peripheral blood.$^{53}$ Removal of the pineal gland (pinealectomy) in
rats significantly reduces both serum and bone marrow concentrations of melatonin
compared to control animals. Exogenous nightly administration of melatonin in drinking
water resulted in a significant increase in bone marrow melatonin compared to control
animals, suggesting exogenous melatonin is sequestered in bone marrow.$^{53}$ Both the MT$_1$
and MT$_2$ melatonin receptors have been found on human osteoblasts.$^{55-57}$ Melatonin
receptor expression on osteoclasts is unknown. The presence of melatonin biosynthetic
enzymes and melatonin receptors in the bone implies a direct role for melatonin in the
bone environment. Melatonin is thought to act on both osteoblasts and osteoclasts to
affect changes in bone tissue. **In vitro**, melatonin stimulates proliferation of extracted
human bone cells\textsuperscript{58} and enhances differentiation of rodent preosteoblast cells into osteoblasts.\textsuperscript{59} Differentiation of hAMSCs into osteoblasts is also enhanced with melatonin through MT\textsubscript{2} receptor signaling.\textsuperscript{46, 60} Chronic treatment of hAMSCs with melatonin \textit{in vitro} significantly enhances expression of alkaline phosphatase, an enzymatic marker of stem cell differentiation into an osteoblast, through action of the MT\textsubscript{2} receptor. The greatest increase in alkaline phosphatase was found to be when melatonin receptors were desensitized as measured by a loss in radiolabeled melatonin binding and a reduced ability of melatonin to inhibit forskolin-induced cAMP accumulation.\textsuperscript{46} Melatonin acting through sensitized receptors also induced mRNA expression of osteogenic genes; gene-induction was blocked by treatment with an MT\textsubscript{2} selective antagonist.\textsuperscript{60} These studies demonstrate the capability of melatonin to enhance differentiation of mesenchymal stem cells into osteoblasts and induce transcription of osteogenic genes through the MT\textsubscript{2} receptor.

In addition to promoting bone formation through actions on osteoblasts, melatonin may also regulate osteoclast activity to reduce bone resorption. Bone remodeling is a tightly coupled process. As illustrated in Figure 3, osteoblasts regulate the activity of osteoclasts and bone resorption through the release of RANKL and osteoprotegerin (OPG). RANKL is produced by osteoblasts and binds to RANK receptors on osteoclast precursor cells to stimulate differentiation into mature osteoclasts. The increase in mature osteoclast number leads to an increase in bone resorption. In order to regulate bone loss associated with osteoclast activity, osteoblasts also release a protein called osteoprotegerin which acts as a decoy. OPG binds to RANKL, preventing RANKL from binding to RANK receptors and diminishing maturation of osteoclasts.\textsuperscript{61, 62}
**Figure 3: Control of osteoclast activation by osteoblasts.** Osteoblasts stimulate osteoclast differentiation through release of RANKL. Increase in osteoclast activity leads to an increase in bone resorption. Osteoblasts also regulate OPG release to bind RANKL and prevent RANKL binding to RANK receptors. Figure reprinted from Coetzee M, Kruger MC. Osteoprotegerin-receptor activator of nuclear factor-kappaB ligand ratio: a new approach to osteoporosis treatment? *South Med J.* May 2004;97(5):506-511, Copyright 2004, with permission from Wolters Kluwer Health, Inc.

*In vitro* treatment of mouse preosteoblast cells with melatonin increases the protein osteoprotegerin and decreases RANKL mRNA transcript.63 By increasing release of OPG from osteoblasts, melatonin may indirectly inhibit osteoclast activation and reduce bone resorption. Melatonin, a potent free radical scavenger, may also inhibit bone resorption by neutralizing the free radicals formed by osteoclasts to initiate bone catabolism.52

Several *in vivo* studies demonstrate the association between melatonin deficiency and bone disease. Pinealectomy in chickens induces formation of scoliotic curvature in the vertebral column.64, 65 Treatment of pinealectomized chickens with melatonin reduced the number of animals that developed scoliosis.66 Bipedal ambulation of C57BL/6J mice results in the development of scoliosis. These mice inherently have
almost no circulating melatonin; however, daily injections of melatonin to restore circulating levels prevented scoliosis development in this model.\textsuperscript{67} Interestingly, pinealectomy did not produce scoliosis in non human primates\textsuperscript{68}; however, these animals have the ability to support weight on all four limbs which may reduced stress on the vertebral column. Little human data exists on the effects of melatonin on bone health; although, melatonin is thought to play a possible role in the development of adolescent idiopathic scoliosis (AIS). While circulating melatonin levels were not found to be different in AIS patients\textsuperscript{69}, researchers have found altered melatonin signaling in osteoblasts from AIS patients compared to cells from healthy individuals.\textsuperscript{56, 70, 71} Melatonin had a reduced ability to inhibit forskolin-stimulated increases in cAMP in osteoblast cells isolated from AIS patients.\textsuperscript{70} A subgroup of AIS patients showed an increased coupling of MT2 receptors to G\textsubscript{s} proteins as opposed to G\textsubscript{i} as found in non-AIS patients.\textsuperscript{56} The exact role of melatonin in AIS remains to be discovered, although MT\textsubscript{2}-mediated melatonin signaling in the osteoblasts appears to be a factor in the etiology of the disease.\textsuperscript{72}

Changes in melatonin rhythms either through altered length of exposure to light\textsuperscript{73} or pinealectomy\textsuperscript{74} disrupts markers of bone turnover in rats, underscoring the connection between melatonin and bone metabolism. Melatonin may work to increase bone formation either directly through action on osteoblast differentiation or indirectly through release of OPG to inhibit osteoclast activation and reduce bone resorption. Koyama et al. found that melatonin, at pharmacological doses, increased bone mineral density by 36% in young mice. Bone mass was also enhanced with melatonin as measured by increases in trabecular bone thickness by 19%, and bone volume per tissue volume by 49%.
Melatonin reduced parameters related to bone resorption including osteoclast surface (74% decrease) and osteoclast number (76% decrease), but treatment had little effect on bone formation measures (bone formation rate, mineral apposition rate, osteoid volume), indicating action primarily through inhibition of resorption. Melatonin has also been shown to enhance the amount of newly formed bone as measured by fluorescent labeling in mice injected nightly with melatonin over 21 days. Studies in ovariectomized rats, a model of postmenopausal osteoporosis, demonstrate the bone protective effects of melatonin when adequate amounts of estradiol are given concurrently. For example, ovariectomized rats treated with melatonin nightly in drinking water had an increase in bone area of the spine and increased bone mineral content of the tibia and skeleton. The highest increases were seen in rats treated with a combination of nightly melatonin and 5 day/week injections of estradiol. There are currently no published human studies examining melatonin for the prevention and/or treatment of osteoporosis; however, a recent study of nurses with 20 or more years of night-shift work indicated a 37% increase in hip and wrist fractures compared to those who never worked at night. This information coupled with the knowledge that melatonin rhythms are shifted and melatonin levels are altered in night-shift workers suggests a relationship between altered melatonin levels and increased risk of fractures. Investigation into the bone protective effects of melatonin in humans and its possible use in osteoporosis is warranted.

**Melatonin and Quality of Life**

Aside from potential bone protective effects, melatonin may also improve quality of life through improvements in sleep. Administration of 0.3 or 1.0 mg of melatonin to
health volunteers decreased sleep onset latency and latency to stage 2 sleep as measured by polysomnography; subjects reported no hangover effects the day after treatment.\textsuperscript{80} Indeed, a meta-analysis of sleep studies found that exogenous melatonin administration decreases time to onset of sleep, increases sleep efficiency (percentage of time asleep out of total time spent in bed), and increases the duration of sleep.\textsuperscript{81} Melatonin alleviates insomnia and improves sleep quality in middle-aged to elderly patients without harmful side effects.\textsuperscript{82} Treatment of perimenopausal women with 3 mg oral melatonin nightly for 6 months resulted in significantly improved mood with treatment compared to placebo based on a survey developed by the study investigators. Many women in the melatonin group reported improvements in sleep duration and quality, although results were not statistically significant.\textsuperscript{83} Disturbances in sleep are a common symptom reported by women during the menopausal transition.\textsuperscript{6} As this population is also at higher risk for developing osteoporosis, a supplement such as melatonin may be beneficial at preventing further bone loss while also alleviating sleep disturbances and improving quality of life.

\textit{Melatonin and Reproduction}

Melatonin may enhance quality of life in perimenopausal women through modulation of reproductive hormones. Melatonin has been established as a regulator of reproduction in seasonally breeding animals; however, the role of melatonin in human reproduction is not fully elucidated.\textsuperscript{84, 85} The exact mechanisms and role for melatonin in reproduction may not be known; however, there is evidence melatonin moderates the reproductive cycle at both a neuronal level and at the level of the reproductive organs. Women with hypothalamic amenorrhea, a disease characterized by low GnRH levels, were found to have higher peak melatonin concentrations and an extended duration of
nighttime melatonin release, implicating melatonin in the regulation of GnRH. Indeed, melatonin induces cyclic decreases in steady-state levels of GnRH mRNA levels in GT1-7 neuronal cells, a murine cell line capable of secreting GnRH. The effects of melatonin were blocked by coadministration with the nonselective melatonin receptor antagonist luzindole. In addition to regulating mRNA, melatonin suppresses GnRH secretion in GT1-7 cells. The actions of melatonin on GnRH appear to be mediated through activation of melatonin receptors and the MAPK pathway.

In addition to effects on GnRH, melatonin has also been implicated in modulating of reproductive hormones at the organ level. Melatonin, at concentrations higher than in serum, has been found in the ovary, specifically preovulatory follicular fluid. Melatonin in the follicular fluid has been hypothesized to play a role in reducing oxidative damage and enhancing maturation of mature oocytes for ovulation. Melatonin may also regulate ovarian function through receptor-mediated pathways as melatonin receptor mRNA and binding sites have also been discovered in ovarian granulosa cells. Melatonin receptor mRNA and binding sites have also been identified in human myometrium (uterine smooth muscle) biopsies. In vitro, melatonin sensitizes uterine smooth muscle cells to procontractile signals, suggesting melatonin may play an important role in preparing the uterus for labor.

Additional evidence of a role for melatonin in reproductive function includes the correlations between melatonin and reproductive hormones. In vitro studies demonstrate the ability of melatonin to enhance secretion of progesterone from human granulosa cells and corpus luteum. Conversely, melatonin has an inverse relationship with estrogen such that when estrogen levels are high melatonin levels are low. A
negative correlation between melatonin and FSH levels was found in perimenopausal women, showing a decrease in melatonin as women transition through menopause.\textsuperscript{105} Aside from relationships between melatonin and other hormone levels, melatonin may interfere with the ability of estradiol to mediate responses through the estrogen receptor.\textsuperscript{106, 107} The complexities of how melatonin mediates responses in the reproductive system are not well-understood, but the evidence supports a direct role for melatonin in the human reproductive system.

\textit{Melatonin Supplementation}

Melatonin is widely available as an over-the-counter oral supplement to relieve jet lag and promote restful sleep. Pharmacokinetic studies of oral melatonin administration show a plasma half-life ($t_{1/2}$) of approximately 40-60 minutes with an estimated oral bioavailability of 15-20\%.\textsuperscript{108, 109} Oral melatonin undergoes extensive first past metabolism in the liver, with much being excreted in the urine as 6-hydroxymelatonin sulfate. Distribution of exogenous melatonin into body tissues has yet to be determined. Timing of oral administration is important based on the intended effects of treatment. Burgess et al. found that administration of 3 mg oral melatonin in the early morning resulted in a phase delay while administration in the afternoon caused a phase advance; administration of melatonin around bedtime had the least phase shifting effects.\textsuperscript{110} Different doses of melatonin may need to be timed differently to reduce the likelihood of phase shifting one’s circadian rhythm.\textsuperscript{110} In this study, we utilized a 3 mg dose of melatonin. The effects of melatonin supplementation on bone have not been investigated; therefore, there was no previous data to indicate the dose needed to alter bone physiology. For this study, a dose was chosen that was known to produce a
physiological effect. Melatonin was given at bedtime in order to minimize disruptions in circadian rhythm.

**Research Objective**

Melatonin supplementation has the potential to prevent the bone loss associated with perimenopause as well as relieve symptoms such as sleep disturbances that can negatively impact quality of life. The purpose of this pilot study was to investigate the effects of daily nocturnal melatonin supplementation in perimenopausal women over the course of six months. Bone density and bone turnover markers were measured to determine how melatonin effects bone health. Quality of life was also assessed through validated questionnaires and daily participant diaries. This study was approved by the Duquesne University Institutional Review Board.

**Hypothesis**

Daily nocturnal melatonin supplementation in perimenopausal women will prevent decreases in bone density, regulate serum markers of bone metabolism, and improve quality of life through regulation of sleep and improvement of menopausal symptoms.

**Specific Aims**

1. Assess the feasibility of recruiting perimenopausal women willing to participate in a blinded, randomized clinical study of melatonin versus placebo

2. Assess the effects of melatonin versus placebo on markers of bone health and subject-reported outcomes of quality of life in perimenopausal women
METHODS

Recruitment and Enrollment

Several recruitment strategies were utilized including advertisements in neighborhood and city newspapers, posting of flyers, on-campus advertising via Duquesne University’s DU Daily website, and news features in the Duquesne University Times and DU Alumni magazine. Interested women were instructed to call the study phone number at which time a phone interview was scheduled. During the initial phone screening, the expectations of the study were detailed, and the potential participant was asked questions to determine eligibility. Inclusion criteria consisted of a woman being age 45 or older, experiencing an irregular menstrual cycle, and having had at least one menstrual period in the past six months. These criteria were used to define the potential participant as perimenopausal. If all inclusion criteria were satisfied, the interview proceeded with inquiries regarding exclusion criteria.

Exclusion criteria were developed based on elimination of factors that may impact bone health, quality of life measures, or were of safety concern. Women taking any medication that could potentially alter bone turnover markers were excluded. These medications included hormone therapy or hormonal birth control, prescription medications for thinning bones (current or within past three months), and chronic use of steroid medications (current or within past six months) as glucocorticoid use can cause secondary osteoporosis. Women with a diagnosis of osteoporosis were excluded because we wanted to examine the effects of melatonin on bone in healthy women. Current smokers were excluded as smoking has been associated with decreases in bone mineral density and may impact bone marker status. Hyperparathyroidism is an over
abundance of PTH, which can greatly alter bone turnover; therefore, women with this condition were excluded from participation. Women with multiple myeloma or other cancers were excluded as these diseases can impact bone metabolism and may impact bone turnover markers, especially if the cancer metastasizes to the bone. Other exclusion criteria included use of sleep medication or antidepressants due to effects on quality of life endpoints such as sleep quality and mood. Women with severe sleep apnea or chronic obstructive pulmonary disease were not eligible to participate as these conditions can impact sleep quality and may influence sleep endpoints in the study. Women with uncontrolled high blood pressure or those currently taking medication for blood pressure were excluded due to safety concerns as melatonin has antihypertensive effects. Liver disease was an exclusion criterion because liver impairment may interfere with the bioavailability of orally administered melatonin. Women with severe lactose intolerance were not eligible because all study medication was compounded with lactose.

Those women deemed eligible to participate were asked to schedule an initial visit to the Center for Pharmacy Care at Duquesne University. Potential participants were sent an information packet before their first visit including a welcome letter, directions, a map to the Center for Pharmacy Care, a brochure about bone density testing, an information sheet, and a copy of the informed consent form. The information sheet contained answers to questions potential subject may have including what to expect at their visit, where to park, and how to contact the study team. The information packet was provided to alleviate participant concerns about the study visit and to encourage study participation. Participants were given the informed consent form prior to enrollment to allow women adequate time to read and understand the document. At the first
appointment, participants were screened for bone health using the Achilles Insight ultrasonometer (GE Healthcare). Women with a T-score less than -2.0 were not enrolled in the study and were advised to follow-up with their primary care physician. Three blood pressure readings were also taken to ensure the participant had an average blood pressure below 140/90 and above 100/60. Women fulfilling the bone density and blood pressure requirements were asked to enroll in the study. Informed consent was obtained from all study participants. This study was approved by the Duquesne University Institutional Review Board.

Randomization, Blinding, Allocation Concealment, and Treatment

Randomization: A total of 20 perimenopausal women were desired for this study. An initial power analysis calculated with a sample size of 10 per group, our study would have 80% power to detect an estimated difference in serum bone markers between groups of 0.89. The sample sizes were modified after a research team meeting with all investigators. As this was a pilot study and we were primarily interested in how melatonin induces changes over six months, the decision was made to randomize more individuals in the treatment group (3:1 ratio of treatment to placebo). This distribution would allow us to obtain an estimate of the effect of the treatment as precisely as possible. Enrolled participants were assigned to receive either placebo (n = 5) or 3 mg melatonin (n=15) treatment based on a computer generated randomization scheme. The actual group assignment was performed using a computerized random number generator.

Blinding: In the informed consent form, subjects were told they would be randomized to one of two treatment groups. Subjects were blinded as to which treatment group they were assigned. Additionally, principal and co-investigators had no knowledge
of group assignments throughout the study period, thus ensuring a double-blinded design. Investigators were unblinded after all participants had completed follow-up and analysis had been performed.

*Allocation Concealment:* Steps taken to ensure allocation concealment consisted of the primary and co-investigators having no knowledge of the next treatment assignment for each subject. The total time to recruit study groups was approximately eighteen months. The research coordinator kept the study subject identification number and their group assignment in a secure location. Only the research coordinator was privileged to this information as subjects entered the study. Additionally, since the primary outcome was a numerical measurement obtained from a blood sample, there was no bias with respect to the research coordinator knowing the assignment.

*Treatment:* Identical placebo and melatonin capsules (Figure 4) were formulated using lactose or 3 mg melatonin in lactose respectively and packaged into individual 30-day blister packs by Avalon Pharmacy (Avalon, PA).

*Figure 4: Study medication in identical blister packs.*
Compounding of capsules was necessary to keep the formulation consistent between placebo and melatonin capsule as well as ensures the placebo contained identical ingredients with the exception of melatonin. Three milligrams is the commonly available supplemental dose of melatonin in the United States. There is no precedence for what dose of melatonin is necessary for effects on bone; therefore, a 3 mg dose was selected because it is capable of producing a measurable physiological response as evidenced by phase shifts in circadian rhythm with 3 mg oral tablets. The timing of the dose was chosen because melatonin will not cause a significant phase shift in circadian rhythm if taken at a subject’s usual bedtime.

Enrolled study participants were asked to commit to monthly visits to the Center for Pharmacy Care over a six month period for a total of seven visits (months 0, 1, 2, etc.). Serum markers of bone metabolism show significant changes in approximately 3-6 months in women taking bisphosphonates or teriparatide for osteoporosis; therefore, we felt six months would be a adequate time period to detect changes in markers of bone turnover with melatonin. During the baseline visit (month 0), subjects were asked to complete forms detailing basic demographic information as well as listing all regular prescription and non-prescription drug and/or supplement use. Participants were given a one-month supply of study medication and were instructed to take one pill approximately two hours before bedtime. A daily journal was also provided to collect information regarding compliance, sleep duration, menstrual cycling, physical activity, and any other information the participant deemed relevant to how she was feeling. Participants were asked to bring their empty pill pack as well as their completed diary pages to the next study visit. Diary pages were color-coded by month to facilitate return of completed
portions at each study visit. Empty blister packs were utilized to track compliance with taking study medication. At each monthly visit, three blood pressure readings were obtained by the study nurse to ensure melatonin did not adversely impact blood pressure. Participants were also asked to report any adverse effects at each visit.

**Assessment of Bone Density**

Bone density was measured monthly by ultrasound of the os calcis (heel) using the Achilles InSight ultrasonometer (GE Healthcare, Waukesha, WI). Participants were asked to place their bare foot between the ultrasound membranes. Measurements were taken on the non-dominant foot (e.g., measurement of the left foot in a right-handed dominant person) to account for possible differences in bone density that may result from favoring the dominant foot. The assessment area was sprayed with ethanol to thoroughly wet the foot and membranes to ensure proper signal transduction. During measurement, the membranes filled with warm water to completely surround the subject’s heel. Results were reported as T-scores, a measure of how many standard deviations the ultrasound reading of the participant compares to the reading of a healthy young adult at peak bone mass. A positive T-score indicates bone density above a young adult; a negative T-score indicates bone density less than a young adult. Changes to bone density as measured by ultrasound can take up to a year or more to be detectable. Therefore, monthly changes in bone density were not expected, and only the month 0 and month 6 measurements were used in analyses.

**Collection and Storage of Serum Samples**

Serum samples were collected bimonthly (months 0, 2, 4, and 6) during daytime study appointments. Participants were not required to fast. Blood was obtained by the
study nurse via venous puncture using a BD Vacutainer Safety-Lok blood collection set with a 23 gauge needle. Samples were collected in 8.5 ml BD Vacutainer SST Plus blood collection tubes. Blood was allowed to clot for at least 30 minutes at room temperature before centrifugation at approximately 1200 x g for 15 minutes. Serum was removed from separating gel, aliquoted, and stored at -20°C until use.

**Assessment of Bone Turnover Markers (BTMs)**

In order to investigate changes in bone metabolism, two markers of bone turnover were measured in serum taken at months 0, 2, 4 and 6. Osteocalcin was measured using the N-MID® Osteocalcin enzyme-linked immunosorbent assay (Immunodiagnostics Systems, Fountain Hills, AZ) to monitor formation of new bone by osteoblasts. The osteoclast marker, amino-terminal cross-linking telopeptide of type I collagen (NTX), was used as an indicator of bone resorption. NTX levels were determined using the Osteomark® NTx serum enzyme immunoassay (Inverness Medical, Princeton, NJ). Procedures were carried out according to the manufacturers’ instructions.

Osteocalcin was measured to determine how melatonin treatment affected bone formation. Serum samples were pipetted into individual microplate wells precoated with streptavidin. Two monoclonal human antibodies against osteocalcin – one biotinylated and one peroxidase conjugated – were added to each well and incubated with the sample for two hours. Each antibody recognizes a different amino acid portion of osteocalcin. Osteocalcin in the sample is captured and held to the microplate well by interaction with the antibodies. After a series of washes, a chromogenic agent was added to produce a color in the presence of antibody-bound osteocalcin. The absorbance of each well was
measured to determine the concentration of osteocalcin present. Samples with more osteocalcin had higher absorbance values.

NTX was measured to determine if melatonin had an impact on bone resorption. Serum samples were added to microplate wells containing an adsorbed NTX epitope. A horseradish peroxidase conjugated monoclonal antibody was added to each well. NTX in serum samples competes with the adsorbed NTX for binding sites on the antibody. After incubation, a series of washes were performed to remove unbound sample and NTX in the sample bound to the antibody. A chromogenic agent was added to detect the amount of antibody bound to NTX on the microplate well. The absorbance of each well was measured to determine the concentration of NTX in each sample. A decrease in absorption value indicated a higher serum NTX level.

Concentrations of osteocalcin and NTX were determined using the Perkin Elmer Victor3 1420 Multilabel plate reader with Workout 2.0 software (Waltham, MA). Absorbance readings of standards, controls, and subject samples were measured at 450 nm for both the osteocalcin and NTX immunoassays. A standard curve was generated for each assay using the four parameter logistic curve fit function as recommended by each assay protocol. Sample concentrations were calculated by Workout 2.0 software based on the generated standard curves. Figure 5 illustrates sample standard curves from both the osteocalcin and NTX immunoassay. Additional standard curves can be found in Appendix A.
Serum Melatonin Levels

In order to determine whether baseline melatonin levels were related to treatment outcome, daytime serum melatonin levels were measured for each participant using blood samples taken at the baseline visit. Melatonin was also measured at the end of the six-month study to observe any changes in melatonin levels with treatment. Serum samples were analyzed using the melatonin direct radioimmunoassay kit (IBL International, Toronto, ON). The assay was carried out according to the manufacturer’s instructions. Briefly, serum samples were combined with melatonin $^{125}$I-Tracer and a polyclonal melatonin antibody. After overnight incubation, precipitating antiserum was added and tubes were centrifuged to pellet the precipitate. All liquid was removed via vacuum aspiration. The amount of radioactivity in each tube was assessed using the Perkin Elmer Packard Cobra II Auto-Gamma 5000 Series gamma counter to obtain counts per minute (cpm). The cpm value for non-specific binding (NSB) was subtracted from all other cpm values. The percentage of binding in each tube compared to the 0 pg/ml control tube ($B_o$) was calculated to obtain the ($B/B_o$) % value using the equation:

$$\left( \frac{B}{B_o} \right) \% = \left( \frac{cpm_{sample} - cpm_{NSB}}{cpm_{B_o} - cpm_{NSB}} \right) \times 100$$
A standard curve ranging from 2.5 – 750 pg/ml was generated using a nonlinear curve fit algorithm for one site competitive binding assays (Figure 6, GraphPad Prism version 5.01). Melatonin serum concentrations were determined by taking the antilog of the log melatonin concentration corresponding to the sample (B/Bo) % value on the standard curve. Samples with levels below the limit of detection (< 2.5 pg/ml) were assigned a value of 2.5 pg/ml.

![Figure 6: Melatonin RIA standard curve. A standard curve was generated by plotting (B/Bo) % versus the log of the melatonin concentration for each standard. Standard concentrations ranged from 2.5 pg/ml to 750 pg/ml melatonin.](image)

**Menopausal Quality of Life**

Participants were asked to complete the Menopause-Specific Quality of Life – Intervention (MENQOL) questionnaire at both the initial and final study visits. This
validated questionnaire consists of 32 menopausal symptoms divided into four domains: vasomotor, psychosocial, physical, and sexual. The full questionnaire is reproduced at the end of the Methods section. Subjects were asked to indicate if they experienced a specific symptom and, if so, to rate how bothered they were on a scale of 0 (not bothered) to 6 (extremely bothered). Questionnaires were scored according to established guidelines to obtain scores for each of the four domains. Briefly, each survey item was scored on scale of 1 to 8 with 1 corresponding to a “no” answer regarding the symptom and 8 corresponding to an answer of “yes” with the maximum “bothered rating” of 6. Domain scores were obtained by calculating the mean of all item scores within that particular domain. The vasomotor domain was composed of items 1-3. The psychosocial domain included items 4-10. Items 11-26 and 30-32 made up the physical domain. The sexual domain consisted of items 27-29. Missing or incomplete data were handled as described. If an entire item was missed on the month 0 questionnaire, the imputed score for that item was determined as the mean of the item score for all participants who responded. This situation was encountered on one survey throughout the study. If the missing item score was on the month 6 questionnaire, then the missing value was calculated as the mean of the item score from all participants in the subject’s treatment group. Incomplete items occurred when a “yes” was indicated but no rating was checked. For this situation, the item score was determined by calculating the mean of the “bothered” ratings from all other “yes” answers within the domain on the subject’s own survey. Incomplete items occurred on only two surveys, one at month 0 and one at month 6 from two different subjects. Differences in domain scores between month 0 and
month 6 were calculated for each participant. Differences in domain scores were compared between the placebo and melatonin treatment groups.

**Sleep Quality**

The Pittsburgh Sleep Quality Index (PSQI) survey\textsuperscript{116} was administered to participants at the month 0 and month 6 study visits to determine the effect of melatonin treatment on sleep. The PSQI is a validated questionnaire used to distinguish between “good” and “poor” sleepers.\textsuperscript{116} Participants answered a series of questions from seven categories which determine overall sleep quality – subjective sleep quality, sleep latency, sleep duration, habitual sleep efficiency, sleep disturbances, use of sleeping medication, and daytime dysfunction. Questionnaires were scored according to published guidelines to obtain a global PSQI score ranging from 0 to 21, with a higher score indicating difficulty in multiple areas affecting sleep quality.\textsuperscript{116} Differences in global PSQI scores from month 0 to month 6 were calculated for each individual. Mean differences were compared between treatment groups.

**Daily Diary**

Participants were given a daily journal to capture information about compliance, use of prescription and non-prescription medications, the number of hours slept each night, menstrual cycling, exercise, and feelings of well-being. Journal entries were utilized to assess changes in menstrual cycling over the course of the study. Each day women were asked to indicate if their period began or ended. Women were excluded from analysis if diaries were not complete enough to determine length of period, number of periods, or both. Two women were excluded from all evaluations of menstrual cycling. One woman was diagnosed with uterine fibroids, and the other subject had
sustained episodes of heavy bleeding such that cycling was not evident in diary information. Journals were also used to estimate the average number of hours slept each month for each participant. Missing values were estimated using mean replacement. The number of nights in which sleep disturbances were recorded by each individual was also examined.

**Statistical Analysis**

Descriptive statistics (means, medians, standard deviations, and various frequency distributions) were used throughout the dissertation to describe the variables collected. To check the randomization was effective, a comparisons of the baseline characteristics between placebo and melatonin participants was performed using Student’s t-test for independent samples (continuous data) and Fishers exact test (categorical data). Treatment differences in continuous variables measured at month 0 and month 6 were analyzed by first calculating the difference between the times for each individual and these individual differences were compared between treatment groups using Student’s t-test with Welch’s correction. This test is equivalent to performing a mixed models analysis with group (fixed effect), subject nested within group (random effect), time (fixed effect) and time by group interaction (fixed) terms in the model and testing the group term within the model. Similarly, continuous variables with repeated measures over time (0, 2, 4, and 6 months) were also analyzed using mixed effects analysis of variance (except time was considered random). Means between groups at each time point were tested using Tukey’s HSD post-hoc multiple comparison procedure. Statistical testing was carried out using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA) and JMP versions 8 and 9 (SAS Institute Inc, Cary, NC).
Results were considered significant at $p < 0.05$. Analysis was performed using the intention-to-treat principle.
Study Advertisements

Are You a Woman Concerned About Thinning Bones?

You may be eligible to participate in a study examining the effect of melatonin on thinning bones in perimenopausal women.

Participants should be:

- Aged 45 and older
- Experiencing an irregular menstrual cycle
- Have had at least one period in the past 6 months

Participation requirements include monthly visits to the Center for Pharmacy Care at Duquesne University School of Pharmacy over a 6 month period.

(Participants in the study will receive compensation for session visits and parking)

Please leave a message at the number listed below and a member of the research study team will return your call and tell you more about the study.

412-396-5874

Investigators: Paula Witt-Enderby, PhD, Duquesne University School of Pharmacy; Hilde Berdine, PharmD, Duquesne University School of Pharmacy; Chris O’Neil, PharmD, Duquesne University School of Pharmacy; Holly Lassila, DrPH, Duquesne University School of Pharmacy; Judith Balk, MD, MPH, Magee Women’s Hospital
About the Osteoporosis Study at Duquesne University

Osteoporosis ("porous bones"), a disorder that causes bones to weaken and break easily, affects about 10 million Americans, 80% of them women. A major risk factor for women developing this skeletal disorder is a drop in estrogen associated with menopause. Current treatment options for these patients include various prescription drug therapies to protect their bones from further loss.

Duquesne University researchers are looking for a safer alternative to preventing bone loss and have discovered positive laboratory results with melatonin. A naturally occurring hormone found in all animals (including humans), melatonin is currently available as an over-the-counter supplement for insomnia, jet lag and depression. Melatonin also has shown promise in producing more of the cells that form bone.

Eligible participants in Duquesne’s Osteoporosis Study will undergo an initial health screening, including a bone density test and blood test. Then they receive either the study medication or a placebo at monthly appointments and will be asked to record their daily activities over a six-month period.

About the Research Team

The principal investigator for the Osteoporosis Study is Paula Witt-Enderby, PhD, professor of pharmacology at the Mylan School of Pharmacy. Her investigative team includes Mylan School of Pharmacy faculty members Christine O’Neil, Pharm D; Hilde Berendonk, Pharm D; Holly Lassila, DPH; and Judith Balk, MD, MPH, a physician at Magee-Womens Hospital of UPMC.

WWW.PHARMACY.DUQ.EDU
Volunteers sought for bone loss study

By Terri T. Johnson
Almanac staff writer
tjohnson@thealmanac.net

As estrogen hormone levels drop in menopausal women, bone loss increases. Two researchers in Pittsburgh are overseeing a clinical study to determine if melatonin may increase formation of bone-forming cells and they are looking for volunteers.

Leading the trail are Dr. Paula Witt-Endersby, professor in the Mylan School of Pharmacy, and Dr. Judith Balk, assistant professor at the University of Pittsburgh School of Medicine’s Department of Obstetrics, Gynecology and Reproductive Sciences and affiliated with Magee-Women’s Hospital.

The study is ongoing and volunteers are encouraged to join at any time. Nine more are needed.

Volunteers must be 45 years old and preferably no older than 68, and with irregular menstrual periods.

Balk said anyone wishing to be screened to be a volunteer cannot have high blood pressure or take any medication for anxiety, high blood pressure, a prescription sleep aid or medication for bone loss, such as Evista.

So far about 70 to 75 women have shown interest with only 11 qualifying, Witt-Endersby said.

Melatonin is available in over-the-counter supplements and mimics the body’s natural hormone producing ability during darkness.

Participating in the clinic trial is very easy, the researchers said. The first visit at the Oakland site is about 40 minutes long. Free valet parking is provided. Future visits are shorter.

“We are very cognizant of people’s lives,” Balk said of the six-month volunteer involvement.

“We have Saturday hours and we are very, very open to making appointments.”

Melatonin is currently used primarily to combat jet-lag symptoms, Witt-Endersby said.

“None of the (OTC) supplements are FDA approved on the way to take the medication like melatonin is in a research study where you’re monitored,” Balk said.

Self-medicating on melatonin is not recommended.

The two researchers are investigating whether supplementing melatonin will improve bone health in women in the beginning stages of menopause.

In addition to determining the effect on bone loss, the study will attempt to discover if there is a change in other symptoms of menopause, such as lack of sleep, anxiety, irritability and hot flashes. Many of the symptoms are connected to the lack of sleep.

“If they sleep better, it makes them feel better, and we’re also trying to protect their bone loss,” Witt-Endersby said.

For more information, go to www.duc.edu/melatonin

“This is all about the quality of life issues with women, and they are particularly susceptible to not feeling good about themselves at this time of life,” Witt-Endersby said.

Article from the South Hills Almanac, December 2009, reprinted with permission from the Almanac.
# Phone Interview Script

## M.O.P.S. Phone Screening

<table>
<thead>
<tr>
<th>PHONE SCREENER NAME</th>
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<tbody>
<tr>
<td>DATE</td>
<td></td>
</tr>
<tr>
<td>PARTICIPANT NAME</td>
<td></td>
</tr>
<tr>
<td>SCREENING ID</td>
<td></td>
</tr>
<tr>
<td>TELEPHONE NUMBER</td>
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<tr>
<td>ELIGIBLE</td>
<td></td>
</tr>
<tr>
<td>DATE OF FIRST VISIT</td>
<td></td>
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<tr>
<td>INFO PACKET SENT</td>
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</tbody>
</table>

How did you hear about this study? ___________________

Thank you for calling to find out more about our research study. My name is _________________. I am calling from the Duquesne University School of Academic Research Center for Pharmacy Care. The purpose of this research study is to examine the relationship between melatonin and the thinning of bones that occurs with age. Specifically, we want to determine if taking melatonin regularly has any effect on certain chemicals in our bodies that reflect the degree at which bone is being made or broken down.

Before enrolling people in this study, we need to determine if they are eligible. You don’t have to answer these questions if you don’t want to. The purpose of these questions is only to determine whether you are eligible for our study. Remember, your participation is voluntary, you do not have to complete these questions. Do I have your permission to ask you these questions?

{If NO} : Thank you very much for calling.

{If YES} : As part of our study, we will be asking women age 45 and older who have had at least one period in the last 6 months to participate. The study duration is 6 months. Participation in this study requires monthly visits to the Center for Pharmacy Care at Duquesne University. At each visit, we ask that you have your bones screened for their health status. We will also collect small blood samples four times throughout the study to measure the markers of bone turnover. On your first and last visits, you will be asked to fill out two short questionnaires related to your quality of life, menopausal symptoms, and sleep habits. You will also be asked to keep a daily journal throughout the study. After completion of the study, you will be compensated for your time and involvement. Do you think you might be interested in participating in this study?

{If NO} : Thank you very much for calling.

{If YES} : I am now going to list some conditions that help us determine your eligibility for the
study. All that we ask is that you listen to the conditions and then indicate if they apply to you.

Do I have your permission to continue?

**{IF NO}**: Thank you very much for calling.

**{IF YES}**: Please feel free to ask if you need me to repeat or clarify anything.

<table>
<thead>
<tr>
<th>Question</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Are you age 45 or older?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are you experiencing an irregular menstrual cycle that is not due to an underlying condition such as polycystic ovarian syndrome or hypothyroidism?</td>
<td></td>
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<tr>
<td>Have you had at least one period in the last 6 months?</td>
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</tbody>
</table>

**{IF NO to any}**: Unfortunately, you are not eligible to participate in this study. I appreciate your interest and thank you very much for your time.

**{IF YES to all}**: Continue with questions

<table>
<thead>
<tr>
<th>Question</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Are you currently using any hormone replacement therapy or hormone birth control such as oral medications, creams, gels, patches, vaginal suppositories, or injectables?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Have you been diagnosed with osteoporosis?</td>
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<td></td>
</tr>
<tr>
<td>Are you currently taking medications for thinning bones such as calcitriol or bisphosphonates (Fosamax, Actonel, Boniva, Aredia, Reclast, Evista)?</td>
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</tr>
<tr>
<td>- Have you taken any of these types of medications in the past three months?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are you currently using any oral or IV steroid medications, or have you used them in the past 6 months?</td>
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<tr>
<td>Examples: prednisone, cortisone, medrol dose pack, prednisolone</td>
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<tr>
<td>Are you currently taking any prescription sleep aids?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Examples: ramelteon (Rozerem), eszopiclone (Lunesta), zolpidem (Ambien), zaleplon (Sonata), triazolam (Halcion)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are you currently taking any medication for depression?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are you currently taking any medication to control blood pressure?</td>
<td></td>
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</tbody>
</table>
Examples: atenolol (Tenormin), metoprolol (Lopressor, Toprol-XL), or propranolol (Inderal)

Do you have high blood pressure that is not under control? □ Yes □ No
Do you have any condition associated with your liver? □ Yes □ No
Do you have medical conditions such as hyperparathyroidism, cancer or multiple myeloma? □ Yes □ No
Do you suffer from chronic obstructive pulmonary disease (COPD) or severe sleep apnea? □ Yes □ No
Are you lactose intolerant (stomach upset after eating dairy)? □ Yes □ No
Do you currently smoke? □ Yes □ No

[If YES to any] : Thank you for answering these questions. Unfortunately, you are not eligible to participate in the study. I appreciate your interest and thank you very much for your time.

[If NO] : Thank you for answering these questions. Based on the information you have provided, you may be eligible to participate in this study. If you would like to participate, there is additional information that I would like to send you which explains more about the study. I’d also like to set up a time for us to meet at Duquesne University’s School of Center for Pharmacy Care. If you decide that you wish to take part in this study, you will be able to sign up at that time. Keep in mind you are under no obligation to participate in this study. If you decide to participate, it does require monthly visits.

Do you plan on being the area for the next 6 months? __________
Do you have any questions? ____________________________________________

Would you like me to send you more information and set up an initial visit to the Duquesne University Center for Pharmacy Care?

[If NO] : I thank you for your time.

[If YES] : May I have your address?

_________________________________________
_________________________________________

Scheduled appointment: Date ___________ Time ___________

Thank you for your time and I look forward to speaking with you in person.
January 1, 2008

Ms. X
1234 Main Street
Pittsburgh PA 15234

Dear Ms. X:

Thank you for your interest in the Melatonin Osteoporosis Prevention Study at Duquesne University. Enclosed you will find information about the study and your upcoming visit including a map of our campus and directions to the Duquesne University Center for Pharmacy Care. A copy of the research study consent form is also included for you to read before your visit. Please do not sign this form. After we confirm your eligibility for the study, we will go over it with you at your appointment and answer any questions you may have regarding your participation before you are enrolled.

We look forward to meeting you at your appointment on Tuesday, January 14th at 10:00 a.m. If you decide you are no longer interested or if you have any questions or concerns, please do not hesitate to contact us at 412-396-4296.

Sincerely,

Paula A. Witt-Enderby, Ph.D.
Principal Investigator
Professor of Pharmacology
Graduate School of Pharmaceutical Sciences
Duquesne University
Questions About Your Visit

What will happen during my appointment?
You will be asked to undergo a test that measures your heel bone density using an ultrasound machine. There is no pain associated with this procedure. Your blood pressure will also be taken. If it is determined that you are eligible to participate in the study and you are willing to do so, you will be asked to sign a consent form to enroll. You will then fill out personal information sheets, two short questionnaires, and have a blood sample drawn. You will be given a one-month supply of your study medication, a daily diary to take with you, and instructions for participating in the study.

How long will my appointment last?
Your first visit will last approximately 30-45 minutes. If you are enrolled in the study, your subsequent monthly visits will be shorter.

Where should I park?
Parking is in the Forbes Avenue Garage located on Forbes Avenue between McAnulty/Chatham Square and Magee St. Directions to Duquesne are included with this mailing.

Will parking be validated?
You will not have to pay for parking. Take a parking ticket when you enter the Forbes Garage. At your visit, you will be given a parking validation sticker.

Where do I go when I get to Duquesne?
After parking in the Forbes garage, take the elevator to the ground floor (Forbes Ave). Follow the highlighted path on the enclosed campus map across Forbes Avenue, heading toward Fifth Avenue. You will pass Barnes & Noble bookstore on your right side. Our offices are located on the corner of Fifth Avenue and Chatham Square at 1000 Fifth Avenue. The entrance is off of Fifth Avenue.

What should I bring?
Please bring a list of any medications, including the name and dose, which you are currently taking. This includes both prescription and non-prescription medications (such as herbal therapies, supplements).

Do I have to participate in the study?
Participation in this study is voluntary. If you decide before your appointment that you are no longer interested, please call 412-396-1296 to cancel your appointment. During your visit, it may be determined that you do not fit the criteria to participate and therefore would not be enrolled. If you are eligible to participate, you may decide at your appointment whether or not you would like to be enrolled.
CONSENT TO PARTICIPATE IN A RESEARCH STUDY

TITLE: Assessing The Efficacy of Melatonin on Bone Health in Perimenopausal Women

INVESTIGATORS: Paula A. Witt-Enderby, Ph.D., Judith Balk, MPH, MD, Christine O’Neil, Pharm.D., Hilde Berdine, Pharm.D., and Holly Lassila, Dr.P.H.

SOURCE OF SUPPORT: This study is supported by a grant from Duquesne University School of Pharmacy.

PURPOSE: You are being asked to participate in a research project that seeks to investigate if taking melatonin will prevent the thinning of bones or to treat osteoporosis. As part of the study, you will be randomized to receive either melatonin treatment or a placebo (same pill as treatment, but with no melatonin). Randomization is like flipping a coin; we cannot control the outcome of randomization. Of the 20 people participating, 15 of them will be randomized to receive melatonin treatment and 5 of them will receive placebo. At your initial visit, we will be taking a blood sample to measure markers of bone turnover and to determine your circulating melatonin levels. This tells how “active” your bone metabolism is and what your starting melatonin levels are. Each month you will need to visit the lab in order to pick up your monthly supply of pills. At this time, we will be reviewing your diaries and we will also be measuring the density of your heel bone using a machine that causes no pain. You will just put your foot inside machine, and it will automatically measure your bone density. Your blood pressure will be monitored throughout the study. Three blood pressure measurements will be taken at each visit. At months 2, 4, and 6 of the study, we will be drawing another sample of blood. We also want to know how you are feeling and how well you are sleeping so we will be having you fill out two questionnaires at your first and last visits. These are the only requests that will be made of you.

RISKS AND BENEFITS: The majority of people can take melatonin safely. You may experience daytime drowsiness, headache or changes in mood. Additional risks involved in participating in this study include discomfort, bruising, fainting, bleeding or infection due to the blood draw and discomfort related to blood pressure measurement and/or answering a quality of life questionnaire. We will be monitoring you every step of the way. We will have an RN on staff that will be drawing your blood and a physician reviewing your records for
any adverse effects. If you experience any adverse effects, then we will notify you immediately and you may need to seek medical attention through your primary care physician. We will follow up with you and your primary care physician to make sure that you are okay and able to continue in the study. You will be financially responsible for these visits. If you need to seek immediate emergency care, then you will be asked to proceed to the closest emergency department. The costs of the emergency care will be billed to your insurance and what is not covered, if found to be due to the study drug or study procedures, will be paid by the study. The benefits to you participating in this study will be that you will have your bone health checked for free. You may sleep better and have better control over your menopausal symptoms. You will also be part of a very important study that may result in using a drug therapy that will help a lot of women.

COMPENSATION: You will be compensated during and after the study. You will receive $10 per visit to the lab as well as parking validation. You will also be given $25 if you complete the study. Your participation in the project will require no monetary cost to you.

CONFIDENTIALITY: Your name will never appear on any survey or research instruments. No identity will be made in the data analysis. All written materials and consent forms will be stored indefinitely in a locked file in the researcher's office for future reference and because it is part of the medical record. Your response(s) will only appear in statistical data summaries.

RIGHT TO WITHDRAW: You are under no obligation to participate in this study. You are free to withdraw your consent to participate at any time.

SUMMARY OF RESULTS: A summary of the results of this research will be supplied to you, at no cost, upon request.

VOLUNTARY CONSENT: I have read the above statements and understand what is being requested of me. I also understand that my participation is voluntary and that I am free to withdraw my consent at any time, for any reason. On these terms, I certify that I am willing to participate in this research project.

I understand that should I have any further questions about my participation in this study, I may call Dr. Paula Witt-Enderby at 412-396-4346, Dr. Judith Balk at 412-641-5391, Dr. O'Neil at 412-396-6417, Dr. Berdine at 412-396-6422, Dr. Lassila at 412-396-1320 and Dr. Paul Richer, Chair of the Duquesne University Institutional Review Board 412-396-6326.

Participant's Signature ___________________________ Date ___________________________

Researcher's Signature ___________________________ Date ___________________________
Baseline Visit Forms

Consent for Blood Pressure and Bone Density Screening

Blood Pressure and Bone Density Consent and Release Form

The blood pressure assessment is a non-invasive screening using a standard blood pressure cuff and stethoscope. The bone density screening is a non-invasive procedure using an Achilles Ultrasound Instrument. Screening results will provide an estimate of bone strength in the heel. The procedure will require one to be seated, but remain still. One should not experience any discomfort. I am required to place my bare non-dominant heel in the foot well of the Achilles instrument. I understand the instrument used for this screening has been approved by the FDA for general clinical use. I understand that participation in this screening procedure will not prevent me from having osteoporosis.

I hereby release any and all claims I or anyone claiming by or through me, now have or may hereafter acquire against The DUQUESNE UNIVERSITY SCHOOL OF PHARMACY students or staff, and any other person or organization connected in any way with the SCREENING for any and all damages or injuries resulting from or arising out of my participation in the SCREENING or any services provided in connection with this screening.

I understand, by voluntarily requesting and accepting the SCREENING that the results of the type of disease, or any other illness or health condition can only be made by a qualified physician. I also understand the responsibility for having an examination performed by my personal physician to confirm the results of the SCREENING and to obtain advice or treatment is mine alone, and not that of any personal physician. The DUQUESNE UNIVERSITY SCHOOL OF PHARMACY students or staff, or any other person or organization associated with the SCREENING.

I understand that my screening results may be used as an aggregate for the purposes of study, comparison or teaching by The DUQUESNE UNIVERSITY SCHOOL OF PHARMACY students or staff. I understand that my records will not in any way be directly associated with my individual identity and that absolute confidentiality of my entire record will be maintained at all times. I understand that by providing contact information I may be contacted by DUQUESNE UNIVERSITY SCHOOL OF PHARMACY LABORATORY staff to verify that I have sought physician advice for abnormal screening results or to determine my interest in other related screenings or programs offered by the DUQUESNE UNIVERSITY SCHOOL OF PHARMACY LABORATORY.

Signature: ________________________ Date: ________________________

Print Name: ________________________ Date of Birth: ________________________

Address: __________________________ Phone: __________________________

City/State/Zip: ________________________ Primary Care Physician: ______________

I acknowledge receipt of this facility’s NOTICE of PRIVACY PRACTICES:
Name/Signature: ________________________

Your BLOOD PRESSURE

<table>
<thead>
<tr>
<th>BP</th>
<th>SBP</th>
<th>DBP</th>
<th>FOLLOW-UP (Check one)</th>
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</thead>
<tbody>
<tr>
<td>Normal</td>
<td>&lt;120 and &lt;80</td>
<td></td>
<td>Continue your good health habits</td>
</tr>
<tr>
<td>Prehypertension</td>
<td>120-139 or 80-89</td>
<td></td>
<td>2 year recheck</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1 year recheck</td>
<td></td>
</tr>
<tr>
<td>Hypertension, Stage 1</td>
<td>140-159 or 90-99</td>
<td></td>
<td>recheck refer win 2 months</td>
</tr>
<tr>
<td>Hypertension, Stage 2</td>
<td>≥ 160 or ≥100</td>
<td></td>
<td>1 month recheck/referral</td>
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<tr>
<td></td>
<td></td>
<td>1 week referral*</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>immediate referral* based on presentation</td>
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Your Heel Bone Density RESULTS: T-score: __________

We recommend that you report the results of this screening to your doctor as indicated below:

☐ Low Risk – Results should be reported to your doctor at your next regular check-up
☐ Moderate Risk – Results should be reported to your doctor
☐ High Risk – Results should be reported to your doctor as soon as possible

The screening results have been discussed with the participant: ________________________ Date: ________________________

I acknowledge receipt of my screening results: ________________________ Date: ________________________

Peripheral bone density scans of the heel are not necessarily representative of other parts of the body. Research does show that there is a very strong correlation between heel measurements and hip/spine measurements; however, the correlation is not absolute.
Baseline Information Form

A. Personal Information

Date: [ ]

First name: [ ] Last name: [ ] M.I.: [ ]

Address: [ ]

City: [ ] State: [ ] Zip: [ ]

Telephone: [ ] -- [ ] -- [ ]

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 experiencing irregular menstrual cycles that are not due to an underlying condition (such as polycystic ovarian syndrome or hypothyroidism)? ❑ Yes ❑ No

Have you had at least one period in the last 6 months? ❑ 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 osteoporosis? ❑ Yes ❑ No

Are you currently taking medications for thinning bones such as calcitonin or bisphosphonates? ❑ Yes ❑ No

Examples: Fosamax, Actonel, Boniva, Aredia, Reclast, Evista

• Have you taken any of these types of medications in the past three months? ❑ Yes ❑ No
Are you currently using any oral or IV steroid medications, or have you used them in the past 6 months?  
- Yes  ❑ No

Examples: prednisone, cortisone, medrol dose pack, prednisolone

Are you currently taking any prescription sleep aids?  ❑ Yes  ❑ No

Examples: ramelteon (Rozerem), eszopiclone (Lunesta), zolpidem (Ambien), zaleplon (Sonata), triazolam (Halcion)

Are you currently taking any medication for depression?  ❑ Yes  ❑ No

Are you currently taking any medication to control blood pressure?  ❑ Yes  ❑ No

Examples: atenolol (Tenormin), metoprolol (Lopressor, Toprol-XL), or propranolol (Inderal)

Do you have high blood pressure that is not under control?  ❑ Yes  ❑ No

Do you have any condition associated with your liver?  ❑ Yes  ❑ No

Do you have medical conditions such as hyperparathyroidism, cancer or multiple myeloma?  ❑ Yes  ❑ No

Do you suffer from chronic obstructive pulmonary disease (COPD) or severe sleep apnea?  ❑ Yes  ❑ No

Are you lactose intolerant (stomach upset after eating dairy)?  ❑ Yes  ❑ No

Do you currently smoke?  ❑ Yes  ❑ No

Have you ever smoked tobacco products?  ❑ Yes  ❑ No

- Yes, how long ago did you stop?   

C. Medications

Are you currently taking any prescription medications?  ❑ Yes  ❑ No

If yes, please list   

Are you currently taking any non-prescription medications (please include vitamins and herbal supplements)?  ❑ Yes  ❑ No

If yes, please list   

________________________________________

________________________________________
Monthly Visit Record Sheets

INITIAL VISIT FORM

NAME (First, Last): ___________________________ Date: _________

ID _________

VISIT CHECKLIST

☐ Explain and obtain signed Center consent form & give Center privacy policy form Initials: ______

☐ Achilles measurement taken (record below) Initials: ______

T-score -2.0 or lower → NOT ELIGIBLE, refer to physician for follow-up

☐ Blood pressure taken (Total of 3 times, record below) Initials: ______

Pressure lower than 100/60 or higher than 140/90 → NOT ELIGIBLE

☐ Explain study and obtain signed Study consent form Initials: ______

☐ Explain compensation; obtain completed Payment form Initials: ______

☐ Baseline Intake & Fracture Assessment forms Initials: ______

☐ Blood sample taken (Time: __________ AM/ PM) Initials: ______

☐ Quality of Life & Sleep questionnaires administered Initials: ______

☐ Diary and study instructions given and explained Initials: ______

☐ Medication supply 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

<table>
<thead>
<tr>
<th>Achilles Measurement</th>
<th>Initials: ______</th>
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<tbody>
<tr>
<td>Blood Pressure: 1) / 2) / 3) / Avg. /</td>
<td>Initials: ______</td>
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<tr>
<td>Melatonin</td>
<td>Date Entered: __________</td>
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<tr>
<td>Osteocalcin</td>
<td>Date Entered: __________</td>
</tr>
<tr>
<td>NTX</td>
<td>Date Entered: __________</td>
</tr>
</tbody>
</table>
MONTH 1 VISIT FORM

NAME (First, Last): _________________________ Date: __________

VISIT CHECKLIST

☐ Achilles measurement taken Initials: _______

☐ Blood pressure taken (Total of 3 times, record below) Initials: _______

☐ Diary pages collected Initials: _______

☐ Pill count performed Initials: _______

Pills remaining from last month: ___________

☐ Next month medication supply 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 ___________ Initials: _______

Blood Pressure: 1) ______/____ 2) ______/____ 3) ______/____ Avg. ______/____ Initials: _______
MONTH 2 VISIT FORM

NAME (First, Last): __________________________ Date: _________

VISIT CHECKLIST

- Achilles measurement taken (record below) Initials: ______
- Blood pressure taken (Total of 3 times, record below) Initials: ______
- Blood sample taken (Time: ________ AM/ PM) Initials: ______
- Diary pages collected Initials: ______
- Pill count performed Initials: ______
- Pills remaining from last month: __________

- Next month medication supply 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

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<td>Initials: ______</td>
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<tr>
<td>Osteocalcin</td>
<td>Date Entered: ______</td>
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<td>NTX</td>
<td>Date Entered: ______</td>
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</table>
MONTH 3 VISIT FORM

NAME (First, Last): ___________________________  Date: __________

VISIT CHECKLIST

☐ Achilles measurement taken  Initials: ______
☐ Blood pressure taken (Total of 3 times, record below)  Initials: ______
☐ Diary pages collected  Initials: ______
☐ Pill count performed  Initials: ______

Pills remaining from last month: __________

☐ Next month medication supply 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

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<td>Initials: ______</td>
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</table>
MONTH 4 VISIT FORM

NAME (First, Last): ___________________________  Date: __________

VISIT CHECKLIST

☐ Achilles measurement taken (record below)  Initials: ______
☐ Blood pressure taken (Total of 3 times, record below)  Initials: ______
☐ Blood sample taken (Time: _______ AM/ PM)  Initials: ______
☐ Diary pages collected  Initials: ______
☐ Pill count performed  Initials: ______

Pills remaining from last month: ______________

☐ Next month medication supply 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 _______________  Initials: ______
Blood Pressure: 1) _____/____  2) _____/____  3) _____/____ Avg. _____/____  Initials: ______
Osteocalcin _______________  Date Entered: __________  Initials: ______
NTX _______________  Date Entered: __________  Initials: ______
MONTH 5 VISIT FORM

NAME (First, Last): _______________________________ Date: __________

VISIT CHECKLIST

☐ Achilles measurement taken Initials: ______
☐ Blood pressure taken (Total of 3 times, record below) Initials: ______
☐ Diary pages collected Initials: ______
☐ Pill count performed Initials: ______
  Pills remaining from last month: __________
☐ Next month medication supply 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

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<td>Initials: ______</td>
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</table>
MONTH 6 VISIT FORM

NAME (First, Last): ____________________________ Date: _________

VISIT CHECKLIST

☐ Achilles measurement taken (record below)  Initials: ______

☐ Blood pressure taken (Total of 3 times, record below)  Initials: ______

☐ Blood sample taken (Time: _______ AM/ PM)  Initials: ______

☐ Quality of Life & Sleep questionnaires administered  Initials: ______

☐ Diary pages collected  Initials: ______

☐ Pill count performed  Initials: ______

Pills remaining from last month: ________________

☐ Parking sticker given (if necessary)  Initials: ______

FOR OFFICE USE ONLY

<table>
<thead>
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<th>Description</th>
<th>Date Entered:</th>
<th>Initials:</th>
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<td>Blood Pressure: 1) __<strong><strong>/</strong></strong>  2) _<strong><strong>/</strong></strong>  3) _<strong><strong>/</strong></strong> Avg. <em><strong><strong>/</strong></strong></em></td>
<td></td>
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<tr>
<td>Melatonin</td>
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<tr>
<td>Osteocalcin</td>
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<tr>
<td>NTX</td>
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</tr>
</tbody>
</table>

What medication did participant believe they were given? ____________________________
Daily Diary Page

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 ______
**MENQOL Questionnaire**

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.

<table>
<thead>
<tr>
<th>Not at all</th>
<th>Extremely bothered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 1 2 3 4 5 6</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>1. HOT FLUSHES OR FLASHES</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
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<tr>
<td>Yes</td>
</tr>
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<table>
<thead>
<tr>
<th>2. NIGHT SWEATS</th>
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<tr>
<td>No</td>
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<tr>
<td>Yes</td>
</tr>
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<td>0 1 2 3 4 5 6</td>
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<table>
<thead>
<tr>
<th>3. SWEATING</th>
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<tbody>
<tr>
<td>No</td>
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<td>Yes</td>
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<table>
<thead>
<tr>
<th>4. DISSATISFACTION WITH MY PERSONAL LIFE</th>
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<tbody>
<tr>
<td>No</td>
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<td>Yes</td>
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<table>
<thead>
<tr>
<th>5. FEELING ANXIOUS OR NERVOUS</th>
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<tbody>
<tr>
<td>No</td>
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<td>Yes</td>
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<table>
<thead>
<tr>
<th>6. POOR MEMORY</th>
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<tr>
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<table>
<thead>
<tr>
<th>7. ACOMPLISHING LESS THAN I USED TO</th>
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<tbody>
<tr>
<td>No</td>
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<td>0 1 2 3 4 5 6</td>
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<table>
<thead>
<tr>
<th>8. FEELING DEPRESSED, DOWN OR BLUE</th>
</tr>
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<tbody>
<tr>
<td>No</td>
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<tr>
<td>Yes</td>
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<td>0 1 2 3 4 5 6</td>
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</table>

<table>
<thead>
<tr>
<th>9. BEING IMPATIENT WITH OTHER PEOPLE</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
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<table>
<thead>
<tr>
<th>10. FEELINGS OF WANTING TO BE ALONE</th>
</tr>
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<tbody>
<tr>
<td>No</td>
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<td>0 1 2 3 4 5 6</td>
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<table>
<thead>
<tr>
<th>11. FLATULENCE (WIND) OR GAS PAINS</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
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<tr>
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<table>
<thead>
<tr>
<th>12. ACHING IN MUSCLES AND JOINTS</th>
</tr>
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<tbody>
<tr>
<td>No</td>
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<td>Yes</td>
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<td>0 1 2 3 4 5 6</td>
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<table>
<thead>
<tr>
<th>13. FEELING TIRED OR WORN OUT</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
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<td>Yes</td>
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<table>
<thead>
<tr>
<th>14. DIFFICULTY SLEEPING</th>
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<tr>
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<table>
<thead>
<tr>
<th>15. ACHES IN BACK OF NECK OR HEAD</th>
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<tr>
<th>16. DECREASE IN PHYSICAL STRENGTH</th>
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56
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<th>MENQOL Questionnaire continued</th>
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<td><strong>17. DECREASE IN STAMINA</strong></td>
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<tr>
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<tr>
<td><strong>18. LACK OF ENERGY</strong></td>
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</tr>
<tr>
<td>No Yes 0 1 2 3 4 5 6</td>
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<tr>
<td><strong>19. DRY SKIN</strong></td>
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<tr>
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<td><strong>20. WEIGHT GAIN</strong></td>
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<tr>
<td><strong>21. INCREASED FACIAL HAIR</strong></td>
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<tr>
<td>No Yes 0 1 2 3 4 5 6</td>
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<tr>
<td><strong>22. CHANGES IN APPEARANCE, TEXTURE OR TONE OF MY SKIN</strong></td>
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<td>No Yes 0 1 2 3 4 5 6</td>
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<td><strong>23. FEELING BLOATED</strong></td>
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<tr>
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<td><strong>24. LOW BACKACHE</strong></td>
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<td><strong>25. FREQUENT URINATION</strong></td>
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<tr>
<td><strong>26. INVOLUNTARY URINATION WHEN LAUGHING OR COUGHING</strong></td>
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<td><strong>27. DECREASE IN MY SEXUAL DESIRE</strong></td>
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<td>No Yes 0 1 2 3 4 5 6</td>
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<td><strong>28. VAGINAL DRYNESS</strong></td>
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<td><strong>29. AVOIDING INTIMACY</strong></td>
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<td><strong>30. BREAST PAIN OR TENDERNES</strong></td>
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<td><strong>31. VAGINAL BLEEDING OR SPOTTING</strong></td>
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<td><strong>32. LEG PAINS OR CRAMPS</strong></td>
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</table>
Pittsburgh Sleep Quality Index


Name ___________________________ ID # __________ Date __________ Age ______

Instructions:
The following questions relate to your usual sleep habits during the past month only. Your answers should indicate the most accurate reply for the majority of days and nights in the past month. Please answer all questions.

1. During the past month, when have you usually gone to bed at night?
   USUAL BED TIME ______________

2. During the past month, how long (in minutes) has it usually take you to fall asleep each night?
   NUMBER OF MINUTES ______________

3. During the past month, when have you usually gotten up in the morning?
   USUAL GETTING UP TIME ______________

4. During the past month, how many hours of actual sleep did you get at night? (This may be different than the number of hours you spend in bed.)
   HOURS OF SLEEP PER NIGHT ______________

For each of the remaining questions, check the one best response. Please answer all questions.

5. During the past month, how often have you had trouble sleeping because you...
   (a) Cannot get to sleep within 30 minutes
      Not during the past month ______ once a week ______ twice a week ______ times a week ______

   (b) Wake up in the middle of the night or early morning
      Not during the past month ______ once a week ______ twice a week ______ times a week ______

   (c) Have to get up to use the bathroom
      Not during the past month ______ once a week ______ twice a week ______ times a week ______

   (d) Cannot breathe comfortably
      Not during the past month ______ once a week ______ twice a week ______ times a week ______

   (e) Cough or snore loudly
      Not during the past month ______ once a week ______ twice a week ______ times a week ______

   (f) Feel too cold
      Not during the past month ______ once a week ______ twice a week ______ times a week ______

   (g) Feel too hot
      Not during the past month ______ once a week ______ twice a week ______ times a week ______

   (h) Had bad dreams
      Not during the past month ______ once a week ______ twice a week ______ times a week ______

   (i) Have pain
      Not during the past month ______ once a week ______ twice a week ______ times a week ______
Pittsburgh Sleep Quality Index continued

(j) Other reason(s), please describe

How often during the past month have you had trouble sleeping because of this?

<table>
<thead>
<tr>
<th>Not during the past month</th>
<th>Less than once a week</th>
<th>Once or twice a week</th>
<th>Three or more times a week</th>
</tr>
</thead>
</table>

6. During the past month, how would you rate your sleep quality overall?

- Very good
- Fairly good
- Fairly bad
- Very bad

7. During the past month, how often have you taken medicine (prescribed or “over the counter”) to help you sleep?

<table>
<thead>
<tr>
<th>Not during the past month</th>
<th>Less than once a week</th>
<th>Once or twice a week</th>
<th>Three or more times a week</th>
</tr>
</thead>
</table>

8. During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?

<table>
<thead>
<tr>
<th>Not during the past month</th>
<th>Less than once a week</th>
<th>Once or twice a week</th>
<th>Three or more times a week</th>
</tr>
</thead>
</table>

9. During the past month, how much of a problem has it been for you to keep up enough enthusiasm to get things done?

- No problem at all
- Only a very slight problem
- Somewhat of a problem
- A very big problem

10. Do you have a bed partner or roommate?

- No bed partner or roommate
- Partner or roommate in another room
- Partner in same room, but not same bed
- Partner in same bed

If you have a roommate or bed partner, ask him/her how often in the past month you have had...

(a) Loud snoring

<table>
<thead>
<tr>
<th>Not during the past month</th>
<th>Less than once a week</th>
<th>Once or twice a week</th>
<th>Three or more times a week</th>
</tr>
</thead>
</table>

(b) Long pauses between breaths while asleep

<table>
<thead>
<tr>
<th>Not during the past month</th>
<th>Less than once a week</th>
<th>Once or twice a week</th>
<th>Three or more times a week</th>
</tr>
</thead>
</table>

(c) Legs twitching or jerking while you sleep

<table>
<thead>
<tr>
<th>Not during the past month</th>
<th>Less than once a week</th>
<th>Once or twice a week</th>
<th>Three or more times a week</th>
</tr>
</thead>
</table>

(d) Episodes of disorientation or confusion during sleep

<table>
<thead>
<tr>
<th>Not during the past month</th>
<th>Less than once a week</th>
<th>Once or twice a week</th>
<th>Three or more times a week</th>
</tr>
</thead>
</table>

(e) Other restlessness while you sleep, please describe
RESULTS

Specific Aim One

The effectiveness of the various recruitment strategies we employed are shown in Table 1. In terms of generating interest in the study, the article published in the Pittsburgh Tribune-Review\textsuperscript{117} on Monday, March 23, 2009 was the most effective; however, none of the women who responded to this article were ultimately enrolled in the study. The low enrollment from this method of advertisement may be due to ambiguity in the article itself, which resulted in more postmenopausal women responding than perimenopausal women. The majority of enrolled participants were recruited through advertisements aimed at the Duquesne University community and by word-of-mouth.

<table>
<thead>
<tr>
<th>Method of Recruitment</th>
<th>Number of Inquires</th>
<th>Number Enrolled in Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>DU Alumni Magazine article</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>DU Daily</td>
<td>13</td>
<td>3</td>
</tr>
<tr>
<td>DU Newspaper article</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Flyer</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>Newspaper Advertisements</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>South Hills Almanac article</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>South Pittsburgh Reporter</td>
<td>3</td>
<td>0</td>
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<tr>
<td>Tribune-Review article</td>
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<td>0</td>
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<tr>
<td>Word-of-mouth</td>
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<td>6</td>
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<td>Other</td>
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<td>0</td>
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<tr>
<td>Unknown</td>
<td>19</td>
<td>0</td>
</tr>
</tbody>
</table>

DU: Duquesne University

The results of the recruitment and enrollment processes are illustrated in Figure 7. Of the 97 respondents, 82 (84.5\%) were reached for a phone screening. Of those completing the phone interview, 34\% did not meet the inclusion criteria of being
perimenopausal and 22% were not eligible based on exclusion criteria. The three most common reasons for exclusion we encountered were use of medication for depression or anxiety (33% of those excluded), being a current smoker (33%), and use of hormone replacement therapy or hormonal contraceptives (22%). Perhaps the largest obstacle faced during recruitment and enrollment were the 13 women who were eligible for the study but for their own reasons chose not to participate. The most common reason for not enrolling was lack of time and not being able to commit to the monthly visits over the six month study period. Information regarding the sample population utilized in this study is provided in Table 2.

<table>
<thead>
<tr>
<th>Table 2: Sample population characteristics</th>
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<tr>
<td></td>
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<tr>
<td>Mean ± SD</td>
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<tr>
<td>---------------------------------------------</td>
</tr>
<tr>
<td>Age (years)</td>
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<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>Serum Melatonin (pg/ml)</td>
</tr>
<tr>
<td>Serum Osteocalcin (ng/ml)</td>
</tr>
<tr>
<td>Serum NTX (nM BCE)</td>
</tr>
<tr>
<td>T-score (calcaneus)</td>
</tr>
<tr>
<td>Vitamin Supplementation</td>
</tr>
</tbody>
</table>

MENQOL
- Vasomotor | 2.2 ± 1.2 | 1.0 – 4.7 |
- Psychosocial | 3.2 ± 1.4 | 1.0 – 5.9 |
- Physical | 2.8 ± 1.2 | 1.2 – 4.9 |
- Sexual | 2.4 ± 2.2 | 1.0 – 8.0 |
- PSQI | 5.1 ± 2.6 | 1.0 – 11.0 |

Mean ± SD; n = 18; BMI, body mass index based on patient-reported height and weight; NTX, amino-terminal cross-linking telopeptide of type I collagen; BCE, bone collagen equivalents; MENQOL, Menopause-Specific Quality of Life; PSQI, Pittsburgh Sleep Quality Index
Figure 7: Flow of recruitment and enrollment. A total of 97 women responded to advertisements. Nineteen women were randomized, but one woman in the melatonin group withdrew after only two days in the study. A total of 5 women in the placebo group and 13 women in the melatonin group were included in all analyses. ITT, intention-to-treat
Specific Aim Two

Out of 97 respondents, a total of 19 women were initially enrolled in the study; however, one subject withdrew after two days. The subject refused to participate in the study, and since no follow-up data was obtained, the principal investigator decided this individual would be removed and not considered part of the study. Thus, the intention-to-treat analysis consisted of five women in the placebo group (all with complete follow-up) and thirteen women in the treatment group (all with complete follow-up). All women self-identified as Caucasian and ranged in age from 45-54.5 years. Baseline differences between groups are illustrated in Table 3. Subjects in the melatonin group were significantly older compared to placebo subjects (50.3 vs 47.5, respectively). The age difference may not be clinically relevant as there is no set age for the perimenopausal stage. The stage of perimenopause (early versus late) may be a more important factor than age. Older women may show biological differences such as more menopausal symptoms if they are in a later stage of perimenopausal. As may be expected with an older perimenopausal population, MENQOL vasomotor domain scores, including items such as hot flashes, were also significantly higher at baseline in the melatonin group (2.5 vs 1.3). All other variables were not significantly different between groups.
Table 3: Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=5)</th>
<th>Melatonin (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>47.5 ± 2.0 (45.4 – 50.7)</td>
<td>50.3 ± 3.0 (45.1 – 54.5)*</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>21.7 ± 3.5 (17.9 – 26.9)</td>
<td>25.7 ± 3.7 (18.2 – 31.1)</td>
</tr>
<tr>
<td><strong>Serum Melatonin (pg/ml)</strong></td>
<td>41.9 ± 27.5 (2.5 – 75.9)</td>
<td>21.1 ± 22.3 (2.5 – 70.8)</td>
</tr>
<tr>
<td><strong>Serum Osteocalcin (ng/ml)</strong></td>
<td>14.1 ± 3.9 (9.5 – 18.4)</td>
<td>11.6 ± 3.8 (3.8 – 17.6)</td>
</tr>
<tr>
<td><strong>Serum NTX (nM BCE)</strong></td>
<td>12.0 ± 4.4 (6.1 – 16.4)</td>
<td>12.4 ± 4.2 (7.3 – 19.3)</td>
</tr>
<tr>
<td><strong>T-score (calcaneus)</strong></td>
<td>-0.8 ± 0.8 (-1.8 – 0.1)</td>
<td>-0.4 ± 1.1 (-1.5 – 1.6)</td>
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<tr>
<td><strong>Vitamin Supplementation</strong></td>
<td>80% (4/5)</td>
<td>69% (9/13)</td>
</tr>
<tr>
<td><strong>MENQOL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Vasomotor</strong></td>
<td>1.3 ± 0.8 (1.0 – 2.7)</td>
<td>2.5 ± 1.3 (1.0 – 4.7)*</td>
</tr>
<tr>
<td><strong>Psychosocial</strong></td>
<td>2.9 ± 0.8 (1.9 – 3.9)</td>
<td>3.3 ± 1.6 (1.0 – 5.9)</td>
</tr>
<tr>
<td><strong>Physical</strong></td>
<td>2.0 ± 0.9 (1.3 – 3.6)</td>
<td>3.1 ± 1.2 (1.2 – 4.9)</td>
</tr>
<tr>
<td><strong>Sexual</strong></td>
<td>2.0 ± 1.4 (1.0 – 4.3)</td>
<td>2.6 ± 2.4 (1.0 – 8.0)</td>
</tr>
<tr>
<td><strong>PSQI</strong></td>
<td>5.0 ± 1.0 (4.0 – 6.0)</td>
<td>5.2 ± 3.0 (1.0 – 11.0)</td>
</tr>
</tbody>
</table>

Mean ± SD (Range); BMI, body mass index based on patient-reported height and weight; BCE, bone collagen equivalents; MENQOL, Menopause-Specific Quality of Life; PSQI Pittsburgh Sleep Quality Index; * p < 0.05, t-test with Welch's correction

Over the course of the six month study period, bone health was monitored using two different methods. Changes in bone density were measured monthly at the calcaneus using the Achilles InSight ultrasound machine (Figure 8). Participants in the placebo group had an average T-score change of -0.02 over six months. Women in the melatonin group had an average change of 0.05. A more positive T-score indicates an improvement in bone density. Melatonin treatment had no significant effect on changes in bone density over the 6 month study period. Appreciable changes in bone density are often not seen until completion of one to two years of treatment; therefore, it would be expected that any effect of melatonin on BMD may not be observable at this early time point. Bone
mineral density T-scores for each individual over the study period can be found in Appendix B.

In addition to bone density, markers of bone metabolism were measured in serum samples collected bimonthly (months 0, 2, 4, and 6) via venous puncture. The osteoclast marker, amino-terminal cross-linking telopeptide of type I collagen (NTX), was used as an indicator of bone breakdown. Osteocalcin was measured to monitor formation of new bone by osteoblasts. Both of these markers have been measured in perimenopausal women.\(^8\),\(^9\),\(^{14}\),\(^{35}\) Additionally, they were stable enough to be stored for assay at a later date; due to our rolling recruitment, samples were not always run immediately but were frozen until enough samples were collected to complete a full immunoassay plate. Changes in bone turnover markers in response to bisphosphonates can be seen as early as three months\(^{32}\); therefore, these markers may provide information about the effect of melatonin on bone at an earlier time than ultrasound. Figure 9 illustrates the changes in

![Figure 8: Bone mineral density changes over six month study. Mean ± SEM; Change from baseline calculated for each individual. Mean changes between placebo and melatonin compared using t-test with Welch’s correction, p < 0.05; n = 5 placebo, n = 13 melatonin.](image-url)
bone turnover markers for both placebo and melatonin treatment groups. No significant treatment effects were found over time using mixed models analyses for either osteocalcin or NTX. Graphs illustrating the changes in bone markers for each participant can be found in Appendix B.

![Graphs showing serum osteocalcin and NTX levels over six month study period.](image)

**Figure 9: Serum osteocalcin and NTX levels over six month study period.** (A) serum osteocalcin (B) serum NTX. n = 5 placebo, n = 13 melatonin. Data expressed as mean ± SEM. At all time points, differences between treatment groups were non-significant for each bone marker as determined by mixed model analyses.

The remodeling and maintenance of bone is a tightly coupled process that requires a balance in osteoclast and osteoblast activities such that breakdown of old bone is coupled with formation of new bone. Current therapies, with the exception of the anabolic agent teriparatide, work by inhibiting osteoclasts to slow breakdown of bone.\(^{22,27}\) Melatonin is unlike standard osteoporosis therapies in that it has the potential to both stimulate osteoblasts\(^{46,60}\) as well as inhibit osteoclast activity.\(^{63}\) Due to its unique action on bone, we wanted to examine the coupling between osteoclast and osteoblast activity in each treatment group. Figure 10 illustrates the changes in osteocalcin and NTX levels over time for the placebo (panel A) and melatonin (panel B) treatment groups. Panel C of Figure 10 shows how the ratio of osteoclast activity to osteoblast activity (expressed at
the ratio of NTX to osteocalcin levels) changes over the study period. Ratios in the melatonin group appear to be trending towards one over time, possibly indicating a more even balance between osteoclast and osteoblast activity as seen in premenopausal women. Also interesting to note is the slight increase in osteocalcin from month 0 to month 2 with melatonin treatment that continued throughout the duration of the study. Also interesting is the slight downward trend of NTX after the two-month timepoint. This pattern is not seen in the placebo group. Thought it is difficult to draw conclusions based on the small sample size, it is possible that melatonin may induce an increase in osteoblast activity, improving bone marker status in perimenopausal women. This idea is also supported by Figure 10C which reveals a time-dependent trend towards an NTX:OC ratio of 1:1 in the melatonin treatment group. Future studies should examine multiple markers of osteoblast activity such as bone-specific alkaline phosphatase to determine if the same pattern occurs in a larger population and with additional bone markers.
Figure 10: Ratio of bone turnover markers by treatment group. (A) Osteocalcin (left axis) and NTX (right axis) levels for placebo group (n = 5) (B) Osteocalcin (left axis) and NTX (right axis) levels for melatonin group (n = 13). Data expressed as mean ± SEM. (C) Ratio of osteoclast to osteoblast activity calculated as the ratio of NTX to osteocalcin serum levels. Bars within each month indicate mean.

Daytime serum melatonin levels were assessed at month 0 to determine if endogenous melatonin levels predicted a response to melatonin treatment as one previous study found a relationship between endogenous melatonin peak levels and response to exogenous supplementation for sleep. Month 6 melatonin levels were measured to detect changes in melatonin levels after treatment. Figure 11 shows the raw data for the
measurement of daytime serum melatonin levels. Several samples, particularly in the melatonin treatment group, were below the limit of detection. Values varied widely at both months 0 and 6, ranging from undetectable to 75 pg/ml. When undetectable samples were assigned a value of 2.5 pg/ml (the limit of detection), the mean (± standard deviation) baseline serum melatonin levels for the placebo and melatonin groups were 41.9 ± 27.5 pg/ml and 21.1 ± 22.3 pg/ml, respectively. Median values at baseline were 49.0 pg/ml in the placebo group and 13.2 pg/ml in the melatonin group. After the six month study period, mean values were 37.1 ± 23.0 pg/ml in the placebo group and 20.1 ± 26.6 pg/ml in the melatonin group. Median values for placebo and melatonin groups at month 6 were 43.7 pg/ml and 2.5 pg/ml, respectively. There is variability in what value to assign samples with levels below the limit of detection. Options could include assigning a value of zero, using the limit of detection measure, or choosing the value midway between. Due to the number of undetectable samples, it may be inappropriate to perform statistical analysis. Looking at the data by individual person (Appendix B), six subjects in the melatonin group had serum levels below the limit of detection at both baseline and month 6 time points. Of the five remaining subjects in the melatonin group, three had values decrease between baseline and month 6 follow-up while two subjects had an increase from baseline to month 6. Overall, it does not appear that melatonin treatment had an effect on daytime serum melatonin levels. Baseline daytime melatonin levels did not appear to predict the degree of bone marker changes; however, daytime melatonin levels may not reflect the peak level of melatonin secreted at nighttime. A 24-hour melatonin profile would be necessary to fully assess a relationship between melatonin levels and degree of response to treatment.
Figure 11: Daytime serum melatonin levels. Serum melatonin levels were determined at months 0 (baseline) and 6 by radioimmunoassay. Several samples had levels below the limit of detection (< 2.5 pg/ml) and are indicated by the ⊗ symbol.

The MENQOL-Intervention survey was administered at month 0 and month 6 to determine the impact of melatonin supplementation on menopausal symptoms. MENQOL vasomotor, psychosocial, and sexual domain scores did not change significantly with melatonin treatment; however, participants taking melatonin had significant improvement in their MENQOL physical domain scores (Table 4) compared to those in the placebo group (-0.6 vs 0.1, respectively). The decrease in MENQOL physical domain score indicates an improvement in physical symptoms with melatonin treatment. Items included in the physical domain included feeling tired or worn out, difficulty sleeping, breast pain, and vaginal bleeding or spotting.
Information in participant diaries was examined to continue exploring the effect of melatonin on physical aspects of the menopausal transition. Menstrual cycle patterns over the six month study period were charted based on information reported in daily diaries. Women were excluded from analysis if diaries were not complete enough to determine duration of period, number of periods, or both. Two women were excluded from all evaluations of menstrual cycling. One woman was diagnosed with uterine fibroids, and the other subject had sustained episodes of heavy bleeding such that cycling was not evident in diary information. Women in the melatonin group reported significantly fewer periods during the six month study (Figure 12A) compared to those subjects in the placebo group (4.3 ± 0.6, n = 10 melatonin vs 6.5 ± 0.3, n = 4 placebo; mean ± SEM) and a correspondingly higher number of days between periods (Figure 12B; 51.2 ± 11.4, n = 10 melatonin; 24.1 ± 0.9, n = 4 placebo), suggesting melatonin may influence menstrual bleeding patterns in perimenopausal women. Average duration of menstrual periods was not different between treatment groups (Figure 12C).

Table 4: Change in MENQOL and PSQI scores after treatment with melatonin or placebo

<table>
<thead>
<tr>
<th></th>
<th>Placebo n = 5</th>
<th></th>
<th></th>
<th>Melatonin n = 13</th>
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<tr>
<td></td>
<td>Baseline</td>
<td>Month 6</td>
<td>Change</td>
<td>Baseline</td>
<td>Month 6</td>
<td>Change</td>
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<td><strong>MENQOL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vasomotor</td>
<td>1.3</td>
<td>1.1</td>
<td>-0.2 (0.4)</td>
<td>2.5</td>
<td>2.9</td>
<td>+0.4 (1.8)</td>
</tr>
<tr>
<td>Psychosocial</td>
<td>2.9</td>
<td>2.7</td>
<td>-0.2 (1.3)</td>
<td>3.3</td>
<td>2.9</td>
<td>-0.4 (1.3)</td>
</tr>
<tr>
<td>Physical</td>
<td>2.0</td>
<td>2.1</td>
<td>+0.1 (0.5)</td>
<td>3.1</td>
<td>2.5</td>
<td>-0.6 (0.8)*</td>
</tr>
<tr>
<td>Sexual</td>
<td>2.0</td>
<td>1.3</td>
<td>-0.7 (1.4)</td>
<td>2.6</td>
<td>2.2</td>
<td>-0.4 (1.2)</td>
</tr>
<tr>
<td><strong>PSQI</strong></td>
<td>5.0</td>
<td>4.0</td>
<td>-1.0 (1.4)</td>
<td>5.2</td>
<td>4.7</td>
<td>-0.5 (2.8)</td>
</tr>
</tbody>
</table>

Mean (SD); MENQOL, Menopause-Specific Quality of Life; PSQI Pittsburgh Sleep Quality Index; * p < 0.05, t-test with Welch’s correction
Figure 12: Menstrual cycling differences between treatment groups. Based on menstrual cycles recorded in subject diaries, subjects receiving melatonin reported significantly fewer periods (A; 4.3 ± 0.6, n = 10 melatonin vs 6.5 ± 0.3, n = 4 placebo; mean ± SEM; p < 0.05) and a greater number of days between periods (B; 51.2 ± 11.4, n = 10 melatonin; 24.1 ± 0.9, n = 4 placebo; p < 0.05). There was no significant difference in the average length of periods between treatment groups (C; 5.9 ± 0.5, n = 9 melatonin; 6.7 ± 0.8, n = 3 placebo; p > 0.05).

The PSQI survey was administered to participants to assess the impact of melatonin treatment on sleep quality. The results of the PSQI survey did not capture a relationship between melatonin administration and improvement in overall sleep quality (Table 4). As part of the daily diaries, participants were also asked to record the number of hours of sleep they received each night. Analysis of participant diaries showed no significant impact on nightly duration of sleep (Figure 13); however, there appeared to be
less variability in the melatonin group. This could be due to the higher number of participants in the melatonin group, or melatonin may be regulating their sleep-wake patterns. Several subjects also recorded incidents of nighttime disturbances or interruptions; one subject in the melatonin group kept extremely detailed records about nighttime disturbances (Figure 13B). Recording sleep interruptions, however, was not a specific entry requested in the daily journal. Therefore, we were unable to determine treatment effects on sleep disturbances because not all participants provided this information.

![Graph A: Average Number of Hours Slept](image1.png)
![Graph B: Number of Nighttime Interruptions](image2.png)

**Figure 13: The impact of melatonin on sleep patterns.** A. The number of hours slept each night was recorded in subject diaries. Analysis using mixed model analysis of variance showed no significant changes with melatonin treatment (p > 0.05); n = 5 placebo; n = 13 melatonin. B. Example of participant tracking the number of nighttime interruptions. Melatonin appears to decrease disturbances over the study period.

Although there was no significant difference in PSQI scores with melatonin treatment, many participants reported stated they were sleeping better indicating an improvement in sleep quality. In fact, 61.5% (8 out of 13) of subjects given melatonin were able to correctly identify their treatment group. When asked why they thought they were given melatonin, subjects reported reasons such as feeling better and sleeping better. Only 15% of melatonin subjects (2 out of 13) incorrectly identified the treatment group.
For those in the placebo group, 60% of subjects (3 out of 5) correctly identified their treatment group based on no change in sleep or relief of menopausal symptoms and 20% (1 out of 5) incorrectly identified their treatment group; the remaining placebo subject was undecided. These results suggest melatonin may be improving some aspect of well-being that could include more regular sleep patterns or improved quality of sleep; however, a larger trial that analyzes specific aspects of sleep quality would be necessary.

Blood pressure was assessed at each monthly visit to detect alterations in blood pressure with treatment. The average of three measurements was recorded. As seen in Table 5 and Figure 14, there appears to be a downward trend in blood pressure readings in both the placebo and melatonin group, possibly indicating more comfort with the study procedures as time progressed. Interesting to note is the change in systolic pressure with melatonin treatment (Table 5; Figure 14B) which appears to show an even greater decrease than with placebo treatment. Treatment with melatonin does not negatively impact blood pressure in our study samples and, in fact, appears to lower blood pressure. Individual blood pressure changes at each month can be found in Appendix B.

**Table 5: Changes in blood pressure after treatment with melatonin or placebo**

<table>
<thead>
<tr>
<th></th>
<th>Placebo n = 5</th>
<th>Melatonin n = 13</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Month 6</td>
</tr>
<tr>
<td>Systolic Pressure (mmHg)</td>
<td>118.0</td>
<td>115.0</td>
</tr>
<tr>
<td>Diastolic Pressure (mmHg)</td>
<td>76.4</td>
<td>70.6</td>
</tr>
<tr>
<td>Pulse Pressure (mmHg)</td>
<td>41.6</td>
<td>44.4</td>
</tr>
</tbody>
</table>

Mean (SD); No significant differences between groups found using t-test with Welch’s correction.
Figure 14: Blood pressure readings after study period. The mean of three blood pressure measurements is displayed for each participant at baseline and after six months. There was an overall decrease in both diastolic and systolic pressure over the study period in both treatment groups. The decrease in systolic pressure appears to be more dramatic with melatonin treatment.
DISCUSSION

The first aim of this study was to assess the feasibility of recruiting perimenopausal women willing to participate in a clinical trial of melatonin versus placebo. An important part of this pilot study was to identify the best strategies for maximizing interest and subject retention. The recruitment process began in August of 2008 and continued through January of 2010. We successfully enrolled 19 women into the study out of a total of 97 interested women. Most enrolled participants were recruited through Duquesne University advertisements, possibly because women were more willing to enroll in a study at an institution with which they were associated. Word-of-mouth was also an effective strategy in enrolling participants. Perhaps, a personal account of what the study is about and what enrollment entails made more eligible women express an interest. Flyers and newspaper advertisements were limited in the amount of information that could be included. We relied on interested women to call us for more details. Recruitment avenues that allowed us to express the rationale and aims for the study were the most effective at generating inquiries about participation. These strategies included detailed articles in a local neighborhood newspaper (South Hills Almanac), the Duquesne University alumni magazine, and the Pittsburgh Tribune-Review. The Tribune-Review article generated the most phone calls about participation; however, many women were not eligible because they were not perimenopausal.

Inclusion and exclusion criteria can negatively impact study enrollment rates even though they are necessary to obtain a specific cohort of study participants. In an article by Panjari et al. 2008, they describe difficulty in recruiting postmenopausal women in Australia willing to participate in a randomized, double-blind, placebo-controlled trail of
oral DHEA. Panjari et al. found many women did not qualify as postmenopausal and were therefore not eligible to participate in their study. Many of the ineligible women in our study did not meet the inclusion criteria for being in perimenopause (34%) despite the three criteria being stated in our advertisements. Thus, one may speculate the ambiguity faced clinically in describing various states during the transition through menopause extends to the lay population as well. Women may not understand the subtle differences in the terms perimenopause and postmenopause. Many women who did not meet our inclusion criteria were already postmenopausal and expressed concerns about being at risk for osteoporosis. The majority of these women heard about our study through an article written in the Pittsburgh Tribune-Review. The article discussed postmenopausal osteoporosis, which may have led primarily postmenopausal women to express an interest in participation. Perimenopausal women may have thought they were not through menopause and therefore did not need to or were not eligible to participate in the study. Women may associate osteoporosis and bone health with menopause, not realizing bone loss begins to occur before they have their last menstrual period.

Indeed, lack of participation in a prevention trial can become an issue as healthy subjects often feel they are not in need of a therapy. Analysis of trials examining effective recruitment strategies have demonstrated that increased education about the intervention and disease state can increase recruitment in randomized trials. To increase recruitment in future studies surrounding bone loss during perimenopause, educational information about when bone loss occurs in the aging process could be beneficial. Also, women need to be aware they could be experiencing bone loss without having symptoms. In this study, eligible women were given a pamphlet in the
information packet with facts about osteoporosis risk, prevention, and how bone density is measured by ultrasound. An additional strategy could be the use of a questionnaire with recruitment materials. Kendrick et al. found that the inclusion of a home safety questionnaire in recruitment mailings increased enrollment in an injury prevention trial compared to mailings without a questionnaire. Future recruitment materials for a study of melatonin and bone health could include a questionnaire designed to educate women on their risk for osteoporosis.

Enrollment difficulties can stem from a variety of situations. In their recruitment of postmenopausal women, Panjari et al. found approximately 55% of women who were eligible after a phone screening declined to attend their first study visit. The two most common reasons for non-participation were the time constraints involved with participation and wariness of adverse effects. In our study, 36% (13 of 36) of eligible women chose not to attend their initial appointment. As with Panjari et al., we found women were unable or unwilling to make the time commitment or were fearful of possible side effects with melatonin administration. The time constraints of work and family obligations may have been factors in the 36% of women who declined to participate in our study. Most of the women we enrolled were working full-time. Many women expressed a need for weekend hours if they were going to commit to participate in the study. Offering Saturday morning hours once a month was a crucial part of our successful recruitment and retention of participants.

Apprehension over taking an unknown treatment (placebo or study medication) can lead some people to decline enrollment in a randomized study. Interviews with people who declined participation in a randomized, placebo-controlled study of
aspirin revealed concerns from one individual about taking a placebo as they were unclear about what was in the placebo. Another person expressed concerns about not being given aspirin when eligibility screenings identified them as a candidate for therapy. In fact, some potential participants opted to go on aspirin therapy themselves without enrolling to ensure they were on therapy and not placebo. During the recruitment process for our study, one woman asked if she could be placed in the placebo group and subsequently declined participation because she did not want to be randomized. An ‘open’ trial design in which subjects are aware of their treatment has been suggested as one method to increase recruitment, however, the disadvantages to having an unblinded trial may outweigh the increase in enrollment. Potential study participants also have concerns about adverse effects from treatment. Educational material about the intervention may be an additional strategy to prevent eligible subjects from declining participation. One woman declined to participate after reading material on a website that claimed melatonin could cause sleepwalking. Two other women expressed concerns about daytime drowsiness as listed in the potential side effects section of the study consent form. We tried to address concerns potential participants may have had about participating in the study by mailing them an informational packet before their first appointment. We hoped to make the process as simple as possible by providing directions, phone numbers, and a summary of what the appointment would entail. The informed consent form was also provided prior to the first appointment to ensure potential subjects had adequate time to carefully read over the risks and benefits of participation and come to the appointment with any questions or concerns. Willingness on behalf of the research staff to discuss any concerns may alleviate apprehension about
treatment; however, some people may still not feel comfortable taking an unknown treatment with risks of adverse effects. Several studies have found monetary incentives\textsuperscript{121, 123} and telephone calls\textsuperscript{123, 124} to be effective strategies for increasing enrollment and retention of study participants. We utilized both of these strategies and found we were successful in retaining all but one study participant who dropped out very early in the study. Participants were also compensated ten dollars for each visit and offered parking validation. Subjects completing the study received an additional fifteen dollars. Compensation was to make up for the travel expenses associated with each monthly visit. All participants were called within one week of their appointments as a reminder; appointments were rescheduled if necessary. Participants were also given appointment reminder cards at their previous visit with the date and time of their next appointment. We had one instance where a participant did not attend her monthly appointment, despite several phone calls. We were able to reach her within a week and have her come in for her appointment. The high retention rate and low number of missed appointments illustrate our ability to enroll and retain study participants utilizing these strategies.

Future studies with a larger sample size may need to employ additional marketing strategies to increase enrollment and decrease the recruitment time period. Through this study, we have identified that the most effective advertisements are those in which more details about the study can be given, including the purpose of the study and the benefits of the intervention. Future strategies targeting perimenopausal women could include presentations to local women’s groups about osteoporosis, articles about the study in local neighborhood newspapers, and more detailed advertisements in city-wide
newspapers. Educational information about osteoporosis risk may draw more interest in study participation, particularly for women who may not think they need to worry about bone health until they are postmenopausal. Information about melatonin should also be included to help alleviate fears about adverse effects. All women in enrolled in our study self-identified as Caucasian. We did not collect information on race from all screened individuals; therefore, we cannot determine if our recruitment strategies successfully attracted a diverse population. Nevertheless, efforts should be made in future studies to enroll a more racial and ethnically diverse population. Strategies to increase diversity could include advertisements at churches and community centers in areas with high minority populations. Additional studies should utilize telephone reminders, compensation for travel expenses, and weekend appointment hours as we found these strategies to be successful in recruitment, enrollment, and retention.

The second aim of this study was to evaluate the effects of melatonin on bone health and quality of life. The primary endpoints were measurements of bone health including assessment of bone density by quantitative ultrasonography and measurement of bone turnover markers (BTMs) in serum. Quantitative ultrasound results revealed no significant changes in bone mineral density (BMD) after the six-month study period in either the placebo or melatonin treatment groups (Figure 8). Ultrasound of peripheral sites, such as the calcaneus, are similar to DXA scan measurements for the ability to discriminate between women with and without a history of hip fracture and osteoporotic vertebral fractures. However, DXA is still the gold standard method for diagnosing osteoporosis. Peripheral BMD measurements can be obtained at the heel, wrist, or finger and have several benefits over DXA including reduced expense,
portability of the machine, and no exposure to radiation. Bone density measurements at peripheral sites are often utilized for screening purposes to identify people for which further examination by DXA are warranted. Although we did not see a significant change in T-score, this was not an unexpected result. Changes in bone density can take a year or more to be observed based on the sensitivity of imaging methods. Bone density measurements provide information about the mass of bone but do not provide information about the bone microarchitecture or the rate of bone remodeling. The rate at which bone is being remodeled is important when considering osteoporosis as part of the disease pathology centers around an imbalance in bone metabolism. One way to assess bone remodeling is to measure biochemical markers of bone turnover. Bone turnover markers can be measured in serum or urine to provide information about the bone metabolism on a molecular level. BTMs are also useful for monitoring how the body is responding to a particular therapy.

In this study, we utilized two markers of bone turnover: osteocalcin to monitor bone formation and NTX to examine bone resorption or breakdown. As illustrated in Figure 9, we found no significant changes in OC or NTX over the study period in either treatment group. BTM expression did not differ between treatment groups at any time point. In Figure 9, osteocalcin appears to increase from baseline to month 2 with melatonin treatment. While not significant, this could be an indication of melatonin stimulating osteoblast activity. With the sample sizes in this study, a difference of 3.2 ng/ml would be necessary to achieve a statistically significant result as determined by mixed model analysis. A sample size of 35 per group would provide enough participants to detect a 3.2 ng/ml change in osteocalcin with 90% power at an alpha of 0.05. A larger
sample size will provide a better understanding of the ability of melatonin to alter BTM levels in perimenopausal women. Although there were no significant changes in BTMs, there was a downward trend in the ratio of NTX to OC with melatonin treatment (Figure 10C). The action of bone-resorbing osteoclasts and bone-forming osteoblasts are tightly coupled. Osteoporosis is caused by a disruption in the balance of bone metabolism such that the action of osteoclasts outpaces osteoblasts, leading to a net loss of bone.\textsuperscript{11} The ratio of a bone resorption marker to bone formation marker could provide information about the coupling between osteoclast and osteoblast activity. We examined the ratio of NTX to OC and found a trend toward a ratio of 1.0 in the melatonin group. Although the individual BTMs measured were not different between treatment group, the downward trend in NTX:OC ratio with treatment indicates melatonin may possibly work to balance the activity of osteoclasts and osteoblasts.

Serum melatonin levels were measured at baseline to determine if they could predict a response to treatment. In a study of the effects of melatonin on sleep and activity in subjects with brain disorders, Laakso et al. found subjects with lower endogenous nighttime melatonin peak levels had a stronger response to treatment with exogenous melatonin.\textsuperscript{118} In our study, we did not see a vast difference in response to supplementation in those with high versus low daytime melatonin levels. For example, a participant with undetectable daytime melatonin levels had smaller changes in BTMs (ID 017, Appendix B) than a woman with higher daytime melatonin (ID 019, Appendix B). Endogenous melatonin levels may indeed be a factor in how a person responds to treatment; however, peak nighttime levels may be the best indicator of low versus high melatonin secretors. Daytime levels may not be reflective of the peak night levels.
Future studies should consider collecting an initial 24-hour salivary melatonin profile in which participants collect saliva samples every 2-4 hours for one day. This information may provide more details into the relationship between endogenous melatonin profiles and responses to exogenous supplementation.

The pattern of change in BTM can depend on the mechanism of action of a specific treatment. For example, antiresorptive therapies like bisphosphonates significantly reduce bone resorption markers early in the treatment period while bone formation markers do not decrease until approximately four weeks later. As bisphosphonates act through inhibition of osteoclast action, the early response is a drop in bone resorption markers. The bone formation markers do not decline until there is an overall decrease in bone remodeling due to the decline in osteoclast activity. BTM changes with anabolic therapy reflect a different pattern. After treatment with teriparatide, bone formation markers significantly increase. Bone resorption markers do not rise significantly above baseline until approximately six months after beginning treatment. The main mechanism of action for each of these therapies determines whether there will be an overall decrease (antiresorptive mechanism) or increase (anabolic mechanism) in both osteoblast and osteoclast markers, reflecting the overall effect of therapy on the bone remodeling process. Melatonin has several possible mechanisms of action on bone including antiresorptive and anabolic actions (Figure 15). As there is no currently available therapy with both antiresorptive and anabolic mechanisms of action, the pattern of BTM changes with this type of treatment is not established. One would speculate that there may be an initial drop in resorption markers due to antiresorptive effects and a rise in formation markers due to anabolic effects,
reflecting the unique mechanisms of action with melatonin. However, if the primary mechanism of action is an anabolic effect through enhancement of osteoblast activity, there may be an initial increase in bone formation markers followed by an increase in resorption markers at later timepoints, indicating an overall increase in bone remodeling. There are no published profiles of BTM changes with melatonin treatment in perimenopausal women. Our study provides a framework illustrating how two markers of bone remodeling, OC and NTX, respond to melatonin supplemenation. A future study should be conducted to investigate a panel of bone markers to determine the most responsive markers to melatonin treatment.

Figure 15: Potential mechanisms of melatonin action on bone. Melatonin has several potential mechanisms through which bone changes can occur including: (a) stimulation of osteoblast differentiation through MT2 receptors; (b,c) increased secretion of OPG from osteoblasts thereby reducing RANKL binding to RANK receptors; and (d) neutralization of free radicals produced by osteoclasts for bone resorption. Figure Copyright © 2011 From Melatonin in the Promotion of Health, 2nd edition by RR Watson. Reproduced by permission of Taylor and Francis Group, LLC, a division of Informa plc.
Currently, there are no standard guidelines regarding the use of bone turnover markers in clinical practice or research. As such, there are no reference standards for bone turnover marker values. Recently, the International Osteoporosis Foundation and the International Federation of Clinical Chemistry and Laboratory Medicine (IOF/IFCC) highlighted the need for reference standards with regards to which bone turnover markers to measure in clinical studies in order to facilitate comparison of results between studies. They have recommended the use of procollagen type I N propeptide (PINP) as a marker of bone formation and carboxy-terminal cross-linking telopeptide of type I collagen (CTX) as a marker of bone resorption based on criteria such as known characteristics of each marker, availability of assay procedures, and requirements for handling and stability. Perhaps consistency in the use of BTMs in clinical studies will facilitate the development of clinical guidelines for collection, measurement, and interpretation of marker changes with treatment.

It is difficult to determine if there is an established pattern of bone turnover marker changes in perimenopausal women. Firstly, there are few prospective, longitudinal studies examining how BTMs change over time in this population. Additionally, the criteria used to classify a cohort as perimenopausal differs among studies. Lastly, the specific biochemical markers studied and the methods of detection vary widely. One prospective, longitudinal study by Hoshino et al. found an increase in serum osteocalcin (bone formation), urinary deoxypyridinoline (DPD, bone resorption), and urinary pyridinoline (PYD, bone resorption) in perimenopausal women; however, measurements were only taken at baseline and after four years. The changes in BTMs in the interim would have aided in establishing a possible pattern of BTM changes.
in perimenopausal women. Also, the perimenopausal group was defined in retrospect as women who began the study in premenopause and completed the study in postmenopause. The length of time each of the women was in the perimenopausal stage could influence how BTMs changed within the four year study period. A second longitudinal study of bone metabolism during the menopausal transition measured three markers of bone resorption (PYD, DPD, and NTX in urine) and two markers of bone formation [plasma OC and serum bone alkaline phosphatase (BAP)] four times in a one year period. Seifert-Klauss et al. found significant increases in OC, PYD, and DPD after twelve months in the perimenopausal group. NTX levels did not change over time in this group but where significantly higher overall compared to premenopausal women. The authors acknowledge their study may include some women who were 'late' premenopausal at the beginning of the study in their perimenopausal cohort based on their classification criteria. A similar longitudinal study published by Rosenbrock et al. in 2002 attempted to illustrate the changes in BTMs over the course of two years. Several markers of bone turnover were measured at 0, 3, 6, 12, and 24 months in premenopausal, perimenopausal, and postmenopausal women. As with Seifert-Klauss et al., this study may have included some women who were 'late' premenopausal at the beginning of the study in their perimenopausal cohort. Over the course of the two-year study, two out of five markers of bone resorption (PYD and DPD) increased over time in the 19 perimenopausal women studied. Only one of the three bone formation markers (OC) increased in perimenopausal women over the course of two years. This study is perhaps the best illustration of how we would have expected the placebo group to respond in our study. The placebo cohort showed no significant change in bone turnover.
over the six month study. Rosenbrock found significant changes over a two year time period\textsuperscript{9} indicating the need for a longer study period in order to detect changes in untreated women over time. We only followed the change in two bone turnover markers for six months. It may take a longer treatment period to fully understand the impact of melatonin on BTMs.

Markers of bone turnover can be utilized in clinical research to make inferences about specific populations, but large biological variability has been an obstacle for moving analysis of BTMs into clinical practice\textsuperscript{128}. The lack of significant differences between the placebo and melatonin treatment groups may be a reflection of the variability between individuals, which is more evident in a pilot study with small sample sizes. Future studies investigating bone turnover marker changes in perimenopausal women should focus on minimizing sources of variation. Controllable sources of variation in BTM include diet and timing of sample collection\textsuperscript{34,129}. In the current study, participants were not required to fast. Depending on the markers investigated, fasting may be an important factor. For instance, serum CTX levels are known to decrease by 20\% after breakfast\textsuperscript{129}. BTM levels can also vary depending on the timing of sample collection; this is particularly true for bone resorption markers as the lowest values occur in the afternoon and evening\textsuperscript{129}. In the current study, the majority of samples were taken in the morning; however, some participants required afternoon appointments resulting in a later blood collection time. Efforts should be made in future studies to standardize the blood collection time, preferably in the morning. Menopausal status is also a source of variance when measuring BTMs. Bone turnover markers are known to increase from premenopause to postmenopause\textsuperscript{34,35,129}. Perimenopausal women present a unique
challenge in that erratic endocrine changes characterize the perimenopausal state, which could possibly influence bone marker expression.

Two cohort studies have compared BTMs between premenopausal and perimenopausal women. A cross-sectional cohort study of women in Australia measured several markers of bone resorption and bone formation in premenopausal, perimenopausal, and postmenopausal women.\(^8\) Out of four bone resorption markers, only one (urinary NTX) was significantly higher in perimenopausal women as compared to premenopausal women. None of the three bone formation markers were different in perimenopausal women as compared to those in premenopause. Interestingly, the authors found a significant correlation with FSH levels and several, but not all, measured markers of bone turnover such that high levels of FSH were correlated with increased BTMs.\(^8\) A second cross-sectional study analyzed serum osteocalcin and urine NTX levels in pre- and early perimenopausal women from the SWAN study.\(^14\) No significant differences in either bone marker between the two cohorts was found; however, in accordance with Ebeling et al.\(^8\), a significant positive correlation was found between FSH and BTM levels.\(^14\) FSH has been proposed to have a possible role in regulation of bone metabolism, possibly through activation of FSH receptors on osteoclasts to stimulate bone resorption.\(^15\) FSH levels increase as women transition through perimenopause into menopause. Our sample population consisted of women in both early and late perimenopause who most likely had a wide range of FSH values. The heterogeneity in FSH levels may contribute to the variability in BTMs between individuals. The variability in FSH levels may be reflective of the type of menstrual cycle occurring in each woman. Anovulatory menstrual cycles, in which no mature oocyte is released,
occur more often in perimenopause than premenopause. The lack of ovulation is thought to result in sustained high levels of FSH. Changes in bone marker expression can differ between ovulatory and anovulatory cycles. Future studies would benefit from measuring FSH levels to consider this factor into the analysis of BTMs changes over time in perimenopausal women.

In addition to primary endpoints of bone health, we also measured secondary endpoints focused on quality of life. The first aspect of quality of life we examined were symptoms related to the menopausal transition. The MENQOL questionnaire was utilized to detect changes in quality of life associated with vasomotor, psychosocial, physical, and sexual menopausal symptoms. Daily journal entries were examined to chart menstrual cycling. Melatonin has previously been examined for its use in the treatment of vasomotor symptoms, but no significant effects were identified. Our study confirms the lack of vasomotor effects as indicated by the lack of significant change in the MENQOL vasomotor domain score (Table 4). Interestingly, we did detect a significant improvement in subjective measures of physical menopausal symptoms with daily nocturnal melatonin supplementation. Subjects taking melatonin also had fewer menstrual periods during the six-month study (Figure 12). Taken together, these results suggest a role for melatonin in improving patient quality of life during the menopausal transition.

Due to fluctuating hormone levels, perimenopausal women experience irregular and often heavy vaginal bleeding. Our study shows melatonin may influence the length of time between menstrual cycles, possibly indicating a restoration of normal cycling patterns. It has been hypothesized that melatonin may restore normal bleeding patterns in
early postmenopausal women. In a double-blind randomized controlled trial, Secreto et al. found more postmenopausal women receiving melatonin treatment (3 mg orally for three months) reported menstrual flow or spotting compared to women who did not take melatonin. The authors speculate melatonin may resume menstrual flow in early postmenopausal women; however, the inclusion criteria for the study included women who had not had a menstrual period in the past six months. Some subjects may technically have been in perimenopause until one year had passed without menstruation. The inclusion of perimenopausal women could explain why some subjects experienced bleeding or spotting. A study by Bellipanni et al. reported resumption of normal menstrual cycles including bleeding and duration in 12 postmenopausal women taking 3 mg oral melatonin nightly. These women were all 1 to 2 years past their final menstrual period. The restoration of normal bleeding patterns in these studies supports our findings that melatonin treatment may regulate menstrual cycling in perimenopausal women. Melatonin may play a role in the regulation of endocrine changes during perimenopause.

The hormonal imbalance in perimenopause is considered hyperestrogen relative to hypoprogesterone. When compared to premenopausal women, perimenopausal women have increased melatonin during the luteal phase, a time when progesterone is also high. Likewise, melatonin levels increase with progestin levels in premenopausal women taking a 3-phase contraceptive pill. While melatonin has a synergistic relationship with progesterone, it has an inverse relationship with estrogen such that when estrogen levels are high melatonin levels are low. These relationships may be explained, in part, by evidence implicating melatonin in the modulation of ovarian function; however, the exact mechanisms are not well-understood. Melatonin
concentrations in the ovary, specifically in preovulatory follicular fluid, are higher than in serum samples indicating a role for melatonin in the ovary. In addition, *in vitro* experiments demonstrate the ability of melatonin to enhance progesterone secretion from stimulated human granulosa cells and corpus luteum. Melatonin receptor mRNA transcripts and binding sites have been discovered on human granulosa cells, suggesting melatonin may influence ovarian function through receptor-mediated events. A recent study investigated the impact of melatonin on serum progesterone levels in women with low luteal phase serum progesterone. Administration of 3 mg oral melatonin nightly during the luteal phase improved serum progesterone levels in 9 of 14 patients; whereas, only 2 of 11 placebo patients had improved progesterone levels. The interplay between melatonin and reproductive hormones points towards a possible role for melatonin in stabilization of the hormonal imbalances that characterize perimenopause. By balancing the fluctuations in hormone levels, melatonin may relieve some of the more bothersome symptoms associated with perimenopause including irregular bleeding patterns as evidenced by the fewer number of menstrual periods experienced by women in the melatonin group (Figure 12). Improvement of physical symptoms associated with perimenopause would enhance quality of life for women going through the menopausal transition.

Analysis of baseline melatonin levels show more women in the melatonin group (11/13) to have daytime serum melatonin levels below 40 pg/ml compared to placebo (2/5). Women in perimenopause undergo periods of high estrogen secretion, which could possibly affect melatonin levels. This idea is supported by an inverse relationship between estrogen and melatonin. Thus, the low melatonin levels in the melatonin-
treated group could indicate higher estrogen levels, suggesting more women in the melatonin group may have been in a hyperestrogen state. Future studies could also measure estrogen to examine how melatonin levels may change with higher or lower estrogen levels.

In addition to irregular bleeding patterns, perimenopause is often characterized by sleep disturbances. Melatonin is well-known for its ability to regulate sleep patterns; therefore, as part of our secondary endpoints, we examined the impact of melatonin on sleep quality. Treatment with melatonin did not significantly change sleep quality as measured by the PSQI survey. This survey is designed to identify “good” and “poor” sleepers. On the 0-21 PSQI score scale, both groups of subjects had a mean starting PSQI score around 5 (Table 3), indicating that the majority of the enrolled participants may have already been experiencing “good” sleep. Therefore, small changes in sleep quality, which may have been evident to the subjects, may not have been captured with this specific sleep questionnaire. As sleep quality was a secondary endpoint of this study, our measures relied on participant records to identify changes in sleeping patterns. We found no change in the average number of hours slept per night with melatonin treatment (Figure 6); however, participants also kept a normal daily routine throughout the study. Thus, the number of hours of sleep may be more a product of normal scheduling rather than a lack of effect by melatonin. A meta-analysis of studies examining the effects of melatonin on sleep found a significant reduction in sleep onset latency, increased sleep efficiency, and increased total duration of sleep with melatonin therapy as measured by objective sleep endpoints. Interestingly, the authors discovered many studies that relied on subjective measure of sleep found melatonin had no efficacy, indicating objective
measures may be necessary to define small but significant changes in sleep quality. Nevertheless, the fact that the majority of melatonin subjects correctly identified their treatment group based on reported improvements in sleep and well-being indicates melatonin may alleviate the sleep disturbances experienced by perimenopausal women. Melatonin may also improve blood pressure in perimenopausal women as evidenced by the decreasing trend in both systolic and diastolic blood pressure over the study period (Figure 14). The exact mechanisms of how melatonin modulates blood pressure are not well-characterized but may involve antioxidant activity, activation of MT2 receptors on the coronary artery and aorta, and regulation of central nervous system areas involved in control of blood pressure. Nighttime melatonin administration has been shown to significantly decrease nocturnal systolic and diastolic blood pressure in healthy women. In this study, we selected women with blood pressure in the normal range as a change in blood pressure was not our primary endpoint. Perimenopausal women with rising blood pressure may further benefit from melatonin supplementation in addition to its effect on physical symptoms of menopause.

This study was designed as a pilot study to examine the impact of melatonin supplementation on bone health with secondary endpoints examining quality of life issues in perimenopausal women. Limitations to this study include small sample sizes, a significantly older population of women in the melatonin group, and heterogeneity in terms of stage of perimenopause and prevalence of menopausal symptoms. Despite the small sample sizes in this study, we detected a significant improvement in MENQOL physical domain scores with nightly melatonin supplementation. Staging of menopause can affect the perception of symptoms common in the menopausal transition. In this
study, it is possible that women were in different stages of the perimenopausal transition (early vs late). Those just beginning perimenopause may have different perceptions of symptoms compared to women who have been in perimenopause for some time. Additionally, women may have had variable responses to the effects of melatonin depending on their hormone profile. As relief of menopausal symptoms was not the primary outcome of this study, subjects were not stratified based on stage of menopause, degree of menopausal symptoms, or hormonal profile. Thus, larger-scale studies with group stratification may further substantiate the findings of this study related to the effects of melatonin on quality of life issues in perimenopausal women. Additionally, we utilized a dose of 3 mg of melatonin in this study. As there are currently no published results regarding melatonin dosage and effects on bone, we chose a dose known to produce a physiologic effect on circadian rhythm. A larger dose may produce a more robust change in bone markers than was seen in this study. Future studies should examine other dosages of melatonin to investigate how an increased dose may impact changes in bone markers.

The main objective of this pilot study was to generate hypotheses and refine study methods for future investigations. We were successful in recruiting perimenopausal women to participate in this study. Retention of enrolled participants was high, most likely because of monthly follow-up phone calls and a lack of adverse effects. Recruitment strategies for future studies should focus on sources of publicity that allow more details of the study to be given such as newspaper articles in local newspapers. We did not find a significant change in biochemical markers of bone turnover with melatonin treatment. We did, however, find a trend in the ratio of NTX to OC, possibly indicating a
balancing of bone remodeling with treatment. In order to better detect changes in markers of bone turnover, future studies should include measurement of several makers of both resorption and formation. Serum samples should be taken in the early morning in fasting participants to minimize sources of variability due to circadian patterns and diet. Melatonin also improved quality of life scores related to physical symptoms of menopause. Interestingly, we did not find an improvement of sleep patterns with melatonin as measured by the PSQI survey. Serum FSH levels should be measured to better classify participants as being in early or late perimenopause. The influence of melatonin on menstrual cycling could be further explored through more detailed questions in the daily journal entries. Information about the extent of menstrual bleeding, symptoms associated with menstruation like breast tenderness, and duration of the menstrual period would allow for more exploration into how melatonin influences menstrual cycling. The use of activity watches (wrist actigraphy) in conjunction with sleep diaries may provide a more detailed account of sleep disturbances experienced by study participants allowing for more objective analysis of the ability of melatonin to improve sleep quality in perimenopausal women. Overall, our study illustrates the potential for melatonin to enhance quality of life in perimenopausal women by reducing physical symptoms associated with the menopausal transition. Melatonin may also protect women from bone loss by maintaining balance between osteoblast and osteoclast activity. A more extensive, longitudinal study into the long-term effects of melatonin supplementation throughout the perimenopausal transition into menopause is warranted.
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APPENDIX A

Additional Standard Curves for Osteocalcin Immunoassays
Additional Standard Curves for NTX Immunoassays
APPENDIX B

Monthly T-Scores – Placebo Group

ID 003

ID 006

ID 014

ID 016

ID 022
Monthly T-Scores – Melatonin Group

ID 002

ID 004

ID 005

ID 007

ID 008

ID 010

ID 011

ID 013
Monthly T-Scores – Melatonin Group (continued)

ID 017

ID 019

ID 020

ID 021

ID 023
Bone Turnover Markers – Placebo Group

- NTX (nM BCE)
- OC (ng/ml)
Bone Turnover Markers – Melatonin Treatment Group

- NTX (nM BCE)  
- OC (ng/ml)

ID 002
- Baseline Melatonin: 70.8 pg/ml

ID 004
- Baseline Melatonin: 33.1 pg/ml

ID 005
- Baseline Melatonin: < 2.5 pg/ml

ID 007
- Baseline Melatonin: < 2.5 pg/ml

ID 008
- Baseline Melatonin: 51.3 pg/ml

ID 010
- Baseline Melatonin: < 2.5 pg/ml

ID 011
- Baseline Melatonin: < 2.5 pg/ml

ID 013
- Baseline Melatonin: 37.2 pg/ml
Bone Turnover Markers – Melatonin Treatment Group (continued)

- NTX (nM BCE)
- OC (ng/ml)

ID 017

Baseline Melatonin < 2.5 pg/ml

ID 019

Baseline Melatonin 26.9 pg/ml

ID 020

Baseline Melatonin 26.3 pg/ml

ID 021

Baseline Melatonin < 2.5 pg/ml

ID 023

Baseline Melatonin 13.2 pg/ml
Melatonin Serum Levels at Baseline and Month 6
Blood Pressure Measurements – Placebo Group

- ● Systolic Pressure
- ▽ Diastolic Pressure
Blood Pressure Measurements – Melatonin Treatment Group

- Systolic Pressure
- Diastolic Pressure

ID 002

ID 004

ID 005

ID 007

ID 008

ID 010

ID 011

ID 013

Month

Blood Pressure (mmHg)
Blood Pressure Measurements – Melatonin Treatment Group (continued)

● Systolic Pressure ▼ Diastolic Pressure