UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

WNT SIGNALING INHIBITOR CHARACTERISTICS ACCORDING TO BONE STATUS, PHYSICAL ACTIVITY LEVELS, AND MUSCLE FUNCTION IN YOUNG AND MIDDLE-AGED PREMENOPAUSAL WOMEN

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

Degree of

DOCTOR OF PHILOSOPHY

By

PRAGYA SHARMA-GHIMIRE
Norman, Oklahoma
2017

WNT SIGNALING INHIBITOR CHARACTERISTICS ACCORDING TO BONE STATUS, PHYSICAL ACTIVITY LEVELS, AND MUSCLE FUNCTION IN YOUNG AND MIDDLE-AGED PREMENOPAUSAL WOMEN

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF HEALTH AND EXERCISE SCIENCE

BY

_____
Dr. Debra A. Bemben, Chair

_____
Dr. Michael G. Bemben

_____
Dr. Rosemary Knapp

_____
Dr. Jason A. Campbell

_____
Dr. Allen Knehans

This work is dedicated to love of my life Amir and Abhana!

Acknowledgements

First and foremost, I would like to express my sincere gratitude to all the participants who participated in this study, without them this project would not have been successful and I wouldn't be writing this dissertation. Being a graduate student, wife and mother this journey was not easy; with this I would like to express utmost gratitude and heartfelt thanks to my adviser Dr. Deb (Debra Bemben) for her tremendous support, guidance, and insightful advice. Her expertise, guidance and belief in me helped me to develop into not only a better researcher but also a better person. She has set an example of excellent researcher, instructor, mentor and a role model.

I would like to thank to all my committee members, Dr. Michael Bemben, Dr. Allen Knehans, Dr. Rosemary Knapp and Dr. Jason Campbell for their invaluable input, and support that bring out the best in this research. I would like to thank to my lab mates Bree, Echo, and Sam for helping with my data collection.

I would like to thank my grandma, dad, mom, and sisters for their unconditional support and encouragement. I will never find enough words to thank my husband, Amir, and daughter, Abhana, for immense patience, inspiration, and endless faith on me to accomplish this goal. Amir, it's been 7 years of amazing life and expecting many more to go! Last but not the least thank you God.

Table of Contents

List of Tables

List of Figures

Abstract

Introduction: Bone metabolism is regulated by the Wnt signaling pathway and is considered to be crucial for skeletal development. There are two glycoproteins that inhibit Wnt function and bone metabolism: sclerostin and Dickkopf-1 (DKK-1). These are novel markers of bone metabolism and could be considered valuable tools to investigate the mechanisms of bone remodeling. Although the mechanism of the Wnt signaling pathway and its inhibitors on bone biology has been well studied, only sparse data are available in humans, especially related to age and physical activity differences in circulating levels of sclerostin and DKK-1.

Purpose: The primary purpose of this study was to compare serum concentrations of sclerostin and DKK-1 in young and middle-aged premenopausal women. These age groups were selected to allow comparisons between women who are still accruing bone mass versus those who have already achieved their peak bone mass. The present study also evaluated the differences in sclerostin and DKK-1 concentrations based on physical activity status {low, moderate, and health enhancing physical activity (HEPA-active)}.This study further analyzed the relationship between bone density variables, jump trials and leg strength with the serum concentrations of sclerostin and DKK-1.

Methods: In this non-randomized cross-sectional research design, a total of 50 young (n=25) and middle-aged (n=25) women participated and completed all protocols. During the first visit, participants completed informed consent and HIPAA forms, and questionnaires on physical activity, calcium intake, and menstrual history. During the second visit, a blood sample was drawn to measure serum levels of sclerostin, DKK-1 and follicle-stimulating hormone (FSH). Participants' body composition (percent body

fat, fat mass, fat-free mass and bone-free lean body mass), areal bone mineral density (aBMD), and bone mineral content (BMC) of total body, lumbar spine, and dual proximal femur was measured with Dual Energy X-ray Absorptiometry (DXA). Participants' non-dominant tibia (4%, 38%, and 66% sites) was measured with peripheral Quantitative Computed Tomography (pQCT). Lower body strength and power were assessed by a two-leg press maximal strength test (1RM) and a vertical jump test.

Results: Sclerostin levels were significantly lower in young women compared to middle-aged women ($p<0.001$). No significant age group and physical activity differences were observed for DKK-1 levels. Sclerostin levels were significantly higher in HEPA-active women compared to low-moderately active women ($p<0.05$). Sclerostin was significantly but weakly positively related with spine L1-L4 aBMD, spine L1-L4 Z-Score, several hip aBMD variables, specifically, right neck Z-Score, left neck Z-Score, right trochanter BMD, right trochanter Z-Score, left trochanter Z-score and total hip right aBMD. DKK-1 also weakly positively related with spine L1-L4 aBMD, spine L1-L4 Z-Score, right and left trochanter aBMD, and right and left trochanter Z-Scores. There was a significant moderate positive relationship between sclerostin and cortical vBMD at 38% tibia. The correlations ranged from ($r=0.24$ to 0.50; $p<0.05$). There were significant but small negative associations ($r=0.27$, $p<0.05$) between time in air and vertical jump height with sclerostin levels.

Conclusions: Sclerostin concentrations were significantly higher in the middle-aged women, which supports a potential role for sclerostin in age-related bone health. The results from the current study found higher sclerostin concentrations in women

participating in a high volume of physical activity. Age group and physical activity status were associated with higher serum sclerostin levels. Although Wnt signaling inhibitors are the negative regulator of bone mass, the findings of a low positive association between sclerostin/DKK-1 with BMD were unexpected. Further, the current findings may provide preliminary data for future research on exercise interventions and Wnt signaling inhibitors.

Chapter 1: Introduction

In the early 19th century, a distinguished English surgeon, Sir Astley Cooper, described osteoporosis as, "*the lightness and softness that (bones) acquire in the more stages of life"… this state of bone favors much the production of fracture.*" Although the osteoporosis concept was previously investigated during the 1770's by John Hunter, the term "postmenopausal osteoporosis" was coined only in 1940. Subsequently, the concept of osteoporosis has come to reflect a continuum, where several pathogenic mechanisms are associated with loss of bone mass and microarchitectural deterioration of bone mass. According to the World Health Organization (WHO, 1994), osteoporosis is defined as a systemic skeletal disease and is characterized by increased bone fragility and a consequent increase in fracture risk. It has been reported that osteoporosis accounts for one of the ten most common diseases, affecting approximately 10 million individuals in the United States. It has also been estimated that every year 1.6 million hip fractures occur, which could increase from 4.5 million to 6.3 million by the year 2050. Therefore, the impact of osteoporosis is associated with significant burden on an individual's quality of life and to the society (Cooper, 1992; Gusi, 2006; Lane, 2006).

Bone mineral density (BMD) is considered a surrogate measure of bone strength as 75-80% of bone strength comes from BMD (Arnaud, 1996; Baran, 1999). Therefore, loss of BMD is associated with loss of bone strength that leads to increased risk for fracture. It has been widely accepted that at any age, the amount of bone mass reflects the post-natal bone growth from birth to adulthood. Therefore, achieving high peak bone mass in early life is important in predicting lower fracture risk in later life (Khosla, 2013).

The clinical diagnosis of osteoporosis and related fracture is determined by dual-energy X-ray absorptiometry (DXA). DXA measures areal bone mineral density (aBMD), bone mineral content (BMC) at specific sites in the body. Further, the diagnosis of osteoporosis is based on comparing the young adult reference population, if the T-score or number of standard deviations from the reference population mean is -2.5 or lower, then the postmenopausal woman is considered to have osteoporosis (Kanis, 1994).

Bone is a dynamic tissue that undergoes continuous remodeling by the process of bone resorption and bone formation via osteoclasts and osteoblasts, respectively (Robling and Turner, 2006). The remodeling process starts with osteocytes sensing the changes in fluid pressure within the bone, which is a response to mechanical loading. The entire coupling process of resorption and formation can take 3-6 months to complete and occurs at the endosteal surface of the bone (Arnaud, 1996). The bone remodeling process is fine-tuned by parathyroid hormone (PTH), calcitonin and sex steroid hormones (Eriksen, 2010). Hormones related to calcium homeostasis can influence osteoblast and osteoclast activity. The decrease in serum calcium levels is associated with the secretion of PTH and vitamin D, which stimulates osteoclast activity. Calcitonin is then released in response to increased calcium levels, which will inhibit osteoclast activity by stimulating osteoblast activity.

It has been well-established that exercise plays an important role in preventing the loss of bone mass. According to Wolff's law, skeletal function changes based on the increased or decreased demands of mechanical loading. Further, Frost (1997) expanded this concept by adding that muscle mass and muscle contraction are required to generate

mechanical loading. Therefore, load bearing or high impact exercise will stimulate mechanical stress above the threshold, an important process for bone formation. Mechanotransduction is considered as the predominant mechanism explaining the influence of exercise on bone health via mechanical stimuli. However, the exact mechanism describing how bone cells detect the mechanical stimuli and control the remodeling process of bone is not clearly understood.

The discovery of the wingless-related integration site (Wnt) signaling pathway provides insight to osteoporosis as well as other skeletal diseases. Wnt is comprised of a family of secreted signaling glycoproteins, which are important for cell proliferation, differentiation, and apoptosis during embryonic development and postnatal bone growth (Kikuchi, 2009). The signal provided by Wnt receptors activates the β-catenin pathway, which further regulates the expression of Wnt target genes (Clevers, 2012). Several glycoproteins that act as Wnt inhibitors fine-tune the Wnt signaling pathway include sclerostin and Dickkopf related protein-1 (DKK-1). Findings from animal models have suggested that the activation of the Wnt signaling pathway influences the expansion of osteoprogenitors, leading to an increase in bone formation. However, Wnt inhibitors, such as sclerostin and DKK-1 secreted by osteocytes, can inhibit the signaling pathway by binding to the lipoprotein receptor-related protein5/6 (LRP5/6) (Manolagas, 2014).

The inhibition of sclerostin and DKK-1 provides a promising approach to preventing the loss of bone mass. Exercise involving high impact loading has provided useful insight into gaining bone mass and muscular strength, compared to unloading or sedentary behavior among healthy individuals. Although DXA is the gold standard for the diagnosis of osteoporosis based on aBMD, this variable is only a surrogate measure

of bone strength. Therefore, the use osteocyte markers such as sclerostin and DKK-1 could provide crucial information for finding risk for fractures (Zagrodna et al., 2016).

It has been well established that mechanical loading reduces the osteocytes' expression of sclerostin, and thus upregulates the Wnt signaling pathway, enhancing the process of bone formation (Frost, 1999; Klein-Nulend, 2013). Both in human and animal models, mechanical loading is considered a crucial modulator for bone adaptation. Exercise appears to positively influence both areal and cortical and trabecular bone formation, especially in premenopausal women (Manolagas, 2014).

Despite the growing knowledge of the biology of the Wnt/β-catenin pathway and its regulation by sclerostin and DKK-1, sparse data are available addressing the importance of physical activity and the body's response to those regulators. Modder et al. (2011) reported on the circulating sclerostin levels in a large population-based study and found that sclerostin levels were significantly higher with aging in both men and women. Similarly, Amrien et al. (2012) conducted a study in healthy adult men and women examining sclerostin levels in relation to physical activity and found that men had significantly higher sclerostin levels than women and physically active individuals had significantly lower sclerostin levels. However, no data are currently available on the influence of physical activity on sclerostin and DKK-1 in young adult women. Also, growing evidence suggests that increased acceleration during muscle contraction provides mechanical forces to stimulate osteogenesis. Only sparse data are available that address neuromuscular performance and sclerostin levels (Macias et al., 2012; Mosti et al., 2015).

Purpose

The primary purposes of this study were to: (1) Compare serum concentrations of sclerostin and DKK-1 in young (20-30 years) and middle-aged (35-45 years) premenopausal women. These age groups were selected to allow comparisons between women who are still accruing bone mass versus those who have already achieved their peak bone mass. (2) Evaluate the differences in sclerostin and DKK-1 concentrations based on physical activity status: low, moderate, and health-enhancing physical activity (HEPA-active). The secondary purposes of this study were to: (1) Evaluate sclerostin and DKK-1 relationships with aBMD and volumetric bone mineral density (vBMD) in young and middle-aged premenopausal women; (2) Evaluate sclerostin and DKK-1 relationships with leg strength; and (3) Evaluate sclerostin and DKK-1 relationships with jump power.

Research Questions

1. Is there a significant difference in sclerostin and DKK-1 serum concentrations between the two age groups of young (20-30 years) and middle-aged premenopausal women (35-45 years)?
2. Is there a significant difference in sclerostin and DKK-1 serum concentrations based on the physical activity status (low, moderate, and HEPA-active)?
3. Is there a significant interaction between age groups (20-30 years and 35-45 years) and physical activity status (low, moderate, and HEPA-active) for sclerostin and DKK-1 serum concentrations?

Hypotheses

1. Sclerostin and DKK-1 serum concentrations will be lower in young women (20-30 years) compared to middle-aged premenopausal women (35-45 years).
2. Women with higher physical activity levels will have lower sclerostin and DKK-1 serum concentrations.
3. There will be no interaction between age and activity, as both age groups will show the same pattern for sclerostin and DKK-1 serum concentrations, which will decrease as physical activity levels (low, moderate, HEPA-active) increase.

Sub-questions

1. Is there a relationship between sclerostin and DKK-1 serum concentrations and aBMD and vBMD in young and middle-aged premenopausal women?
2. Is there a relationship between sclerostin and DKK-1 serum concentrations and jump power and leg strength in young and middle-aged premenopausal women?

Sub-hypotheses

1. There will be a moderate inverse relationship between sclerostin and DKK-1 serum concentrations with aBMD and vBMD in young and middle-aged premenopausal women.
2. There will be a strong inverse relationship between sclerostin and DKK-1 serum concentrations with jump power and leg press in young and middle-aged premenopausal women.

Significance of the study

Due to the paucity of data in the literature, the present study aimed to investigate the levels of sclerostin and DKK-1 in young and middle-aged women (20-30 yrs and

35-45 years). Wnt signaling is crucial in bone development, which can affect peak bone mass. Sclerostin and DKK-1 are potent regulators of the Wnt signaling pathway, which directly bind to Wnt ligands and suppress gene transcription for bone formation.

To date, no study has examined Wnt inhibitors in young and middle-aged population and its association with areal and volumetric bone densities. Therefore, the present study aimed to compare two Wnt signaling inhibitors to understand the bone biology in young versus middle-aged premenopausal women. The selection of these age groups allowed comparisons between women who are still accruing bone mass versus those who have already achieved their peak bone mass. The understanding of Wnt signaling pathway utilized by sclerostin and DKK-1 is still incomplete and measuring those Wnt signaling inhibitors could be helpful to determine the mechanism of peak bone mass and mechanical loading in bone metabolism.

Assumptions

1. Participants were honest and accurate while completing the health screening questionnaire and other questionnaires.
2. Participants were fasted overnight and well hydrated during the time of blood collection.
3. Participants gave maximum effort during 1RM testing.
4. Participants gave maximum effort for each jump trial.

Delimitations

1. This study included healthy women between ages 20-30 years and 35-45 years old.
2. All the participants were from University of Oklahoma, Norman and surrounding areas.

3. Participants with metal implants in spine or hip were excluded from this study.

Limitations

1. Participants who participate in this study were volunteers, and thus may not accurately represent different sub-populations within young and middle-aged premenopausal women.
2. This study was designed as cross-sectional study; hence cause-effect cannot be determined.
3. Extrinsic factors such as dietary intake and genetics were not controlled.

Operational Definitions

1. 1 Repetition Maximum (1-RM) Test: The maximum amount of weight that can be lifted by the individual one time throughout the full range of motion with correct technique (Harter, 1990).
2. Areal BMD: The bone mineral content in one square centimeter of bone and is measured in gm/cm^2 (Bouxein, 2008).
3. Bone Mineral Content: The amount of measured bone mineral in grams (Nattiv et al., 2007).
4. Bucking Ratio: It is ratio of the outer cortex to the cortical thickness and reflect the strength parameter of hip structural analyses. Buckling ratio greater than 10 reflects significant loss of strength (Choui, 2016).
5. Cortical bone: Compact, highly calcified (~90%) bone, predominantly found in the diaphysis of long bones (Burr and Allen, 2014).
6. Cross-sectional moment of inertia: It is the bending stress that occur from neutral or centroid axis (Beck, 2007).

7. Dual–energy x-ray absorptiometry (DXA): the most widely used method in assessing bone mineral content and body composition in adults and children that utilizes low doses of ionizing radiation and provides precise results in the assessment of bone mineral content and body composition in adults and children (Blake, 2013).
8. Jump Power: The measurement of vertical jump against an individual's center of gravity by elevating off the ground from the standstill position in the assessment of neuromuscular performance (Buehring, 2010).
9. Mechanical Loading: The use of skeletal muscle to overpower a set force by stressing the bone at the specific bone site (Frost, 1997).
10. Mechanotransduction: Detection and response of the bone cell to mechanical loading (Turner, 1998).
11. Osteoblast: Type of bone cell responsible for bone formation (Marcus et al., 2013).
12. Osteoclast: Type of bone cell responsible for bone resorption (Marcus et al., 2013).
13. Osteocyte: Type of bone cell responsible for mechanotransduction (Bonewald, 2011).
14. Osteopenia: Normal loss of bone mass with aging and is determined by a BMD T-score between 1 and 2.5 standard deviations below the young adult mean (T-scores -1.1 to -2.4) (Kanis, 1994).
15. Osteoporosis: A condition when aBMD is ≤ 2.5 SD below compared to Caucasian young adult reference population (T-score ≤ 2.5) (Kanis, 1994).

16. Peripheral Quantitative Computed Tomography (pQCT): An imaging technique that assesses volumetric BMD (vBMD), bone geometry and strength of total bone, cortical and trabecular bone of peripheral limbs (Blew et al., 2014).
17. Premenopausal women: Women with no signs of menopause, have all of their reproductive organs and have a regular menstrual cycle most months of the year (Kremer, 2009).
18. Stress-Strain Index (SSI): A cortical density-weighted section modulus of bone, which gives a measure of bending and torsional strength of the diaphyseal sites in bone (Cointry, 2014).
19. Sectional Modulus (SM): SM reflects the strength parameter according to cross-sectional moment of inertia that occurs at the furthest point from the neutral axis (Choui, 2016).
20. Strength Index (SI): It is the measure of hip ability to withstand a fall at the greater trochanter site (Faulkner et al., 2006).
21. Trabecular Bone: Bone that is found at the ends of long bones, in the lumbar spine and intertrochanteric area of the femur, and that is comprised of the loosely organized porous matrix with high metabolic characteristics (Blew et al., 2014).
22. Volumetric bone mineral density (vBMD): The amount of bone mineral (mg) in a cubic centimeter of bone (Hind, 2007).
23. Wnt signaling: A signal transduction pathway where Wnt binding leads to the stabilization of the β-catenin for cell proliferation and differentiation (Westendorf, 2004)

Chapter 2: Review of Literature

The mechanosensory and mechanoresponsive processes in living organisms have provided critical cues in understanding the growth and development of the skeletal system in human beings (Ingber, 2005). Bone is an adaptable tissue that can respond to the strain imposed by mechanical loading, which is important for the development of bone strength.

During the nineteenth century, Julius Wolff proposed that the stress placed on a bone is the major determinant of the bone's architecture and stated that bone possesses the property of functional adaptation. Bone architecture and mass are adaptable to the demands of the mechanical stress placed upon the skeleton (Turner, 1998). Later, Frost expanded upon Wolff's idea and introduced the mechanostat theory, which postulated that a mechanical threshold can control the formation and removal of bone mass from the skeleton. Frost proposed that below a specific threshold of mechanical loading, bone resorption would occur, and above this threshold; when greater peak load is applied bone formation occurs, resulting in the increased bone strength (Frost, 2000).

Bone adaptation to mechanical loading suggests that bone strength is plastic and can be modulated in adults. Bone adaptation is driven by the processes of activation, resorption and formation (Burr, 2014) modeling and remodeling. Further, with experimental evidence, Turner developed the concept of three fundamental rules for bone adaptation: 1) Bone adaptation is driven by dynamic loading; 2) a short duration of mechanical loading is effective for an adaptive response; and 3) bone cells accommodate and become less responsive with routine mechanical loading (Turner, 1998). Therefore, the adaptive nature of the skeleton provides evidence of the

phenotypic influence of mechanical loading, with diminished physical signals resulting in the onset of loss of bone and muscle mass (Ozcivici et al., 2010). Although the exact mechanism of bone adaptation is not clearly understood, the basics of bone physiology and various biochemical events that take place during the bone modeling and remodeling processes can provide some information regarding bone adaptation. Understanding bone turnover could provide crucial information about how dynamic tissue undergoes resorption or formation. Bone turnover is the coupled process that is carried by osteoblasts, the bone forming cells, and osteoclasts, the bone resorption cells. Osteoblasts and osteoclasts differ in morphological and biochemical properties and, in humans, by-products of these markers have been widely used clinically by measuring them in serum, saliva or urine.

According to the Roux principle (Roux, 1895), bone adaptation is achieved under the influence of a cellular process where the signal for the initiation of bone formation is transmitted via mechanotransduction. Recently, exploring the role of osteocytes have gained attention among researchers. One hypothesis suggested that mechanotransduction can be carried out via fluid flow over the osteocytes and cytoskeleton adhesion protein, which are important for the amplification of the strain (Han et al., 2004; Turner, 1998). Another hypothesis postulated by Bell et al. (2008) suggested that an anatomical structure of osteocytes, the cilium, is able to sense the hydrostatic pressure generated by the mechanical loading. There has been considerable progress made to identify the key players in mechanotransduction, of which inhibition of SOST and DKK-1 expression are the most important. It has been well accepted that Wnt pathways transmit mechanical signals in the bone remodeling process via

activation and inactivation of signaling cascades (Klein-Nulend, 2013). Data from animal models have suggested that the absence of mechanical loading leads to an increase in Wnt signaling inhibitors (Kang and Robling, 2015). Therefore, participating in physical activity or exposure to frequent high-impact exercise could aid the signaling pathway in functioning properly. In humans, only a few studies (see below) have been conducted to explain the beneficial aspects of physical activity in attenuating sclerostin and DKK-1 but there is a lack of information addressing this type of loading.

This review of the current literature covers the mechanical loading, structure, and function of bone cells along with mechanotransduction. Additionally, the signal transduction pathway that governs bone metabolism are reviewed. The signal transduction pathway enables bone to withstand different forces or adaptation that depends on the individual biochemical requirements. Sclerostin and DKK-1 are regarded as the most potent regulators of the Wnt signal transduction pathway. Further, mechanotransduction is discussed to provide insight on how bone cells can sense and transmit signals for initiating the bone formation process, and thus the foundation of the signaling pathway as an assessment of bone metabolism. Evidence on mechanical loading and bone adaptation in the context of the bone response to physical activity in young adult women were reviewed in this section.

Structural, Functional and Metabolic Properties of Bone

Bone is a multifunctional connective tissue that protects the vital organs in the body and provides mechanical support for the body and recently its role on endocrine function has also been studied (Burr et al., 2014). Organic matrix, minerals, and water are the major components of the skeleton. The inorganic phase of bone is comprised of

hydroxyapatite, which is made from calcium and phosphate that provide structural support to the bone (Marcus et al., 2013). The organic phase of bone is comprised of type I collagen and a variety of noncollagenous proteins and proteoglycans. It has been suggested that bone-specific proteins are important in regulating bone turnover precursors (Boskey et al., 1998).

The macroscopic structure of bone is comprised of cortical or compact and cancellous or trabecular bone, which are made of the same cells and matrix but have different structural and functional properties. Further, the level of porosity can also differentiate cortical and trabecular bone. The cortical bone, which is 80-90% calcified and 5-20% porous, is mainly found in the diaphysial shaft of the long bones and provides support. The cancellous bone, which is 15-25% calcified and 40-95% porous, is mainly found in the metaphysis of the long bones, lumbar spine, and intertrochanteric area of the femur and provides a metabolic function (Burr, 2014). Quantitative analyses have revealed trabecular thickness and spacing are the major determinants of bone stiffness and strength independent of the bone's porosity (Ulrich, 1999). Aging and disuse patterns are the major contributors of increasing porosity in trabecular bone.

Bone homeostasis is maintained by three types of cellular components known as osteoblasts, osteocytes, and osteoclasts. The accumulation of micro damage and fatigue damage can result in the loss of bone mass if not repaired. The process by which old bone is replaced with new bone is known as bone turnover (Burr, 2014). Bone turnover is a crucial process, as bone is a dynamic living tissue that can undergo the processes of resorption and formation. Bone turnover markers are products released into the blood during formation or resorption processes. The preanalytical variables that influence

bone turnover marker concentrations include circadian variation, menstrual cycle, seasonal variation, fasting versus fed, and physical activity levels (Lombardi et al., 2012). From a clinical perspective, bone turnover marker analyses provide insight into fracture risk and cost-effectiveness of any anti-resorptive therapy. Several methods have been used for measurements of bone-turnover markers, such as radioimmunoassay, immunoradiometric assay, and enzymatic immunoassay.

Bone cells are derived from stem cell lineages known as the mesenchymal lineage and hematopoietic lineage that regulate homeostasis and immune responses (Marcus et al., 2013). The mesenchymal lineage is comprised of bone formation cells, type I collagen, and non-collagenous proteins. Osteoblast differentiation is a multistep cascade of several transcriptional regulatory factors that enhance proliferation, maturation, and termination of osteoblast function. Osteoblasts function to synthesize bone matrix, which is located on the bone surface and cuboidal in shape with enlarged Golgi apparatus. Osteoblasts express alkaline phosphatase (ALP), osteocalcin, type I collagen, and specialized matrix proteins.

The osteoblastic differentiation process is comprised of proliferation, maturation, and termination of the matrix. The expression of alkaline phosphatase (ALP), osteocalcin, type I collagen, and other matrix proteins reflect the rate of bone formation. ALP and osteocalcin are considered as the early and late markers of osteoblast differentiation (Bellido et al., 2014; de Gorter, 2013; ten Dijike, 2013). Osteoblast differentiation involves several important signal transduction pathways through the activation of transcription factor Runx2. Mice deficient in Runx2 completely lack osteoblasts and have skeletons without mineralized matrix. BMP, Wnt,

and Hedgehog are some of the major signaling pathways that promote osteoblast differentiation. Therefore, β-catenin stimulates mesenchymal stem cells to differentiate into chondrocyte precursors, which are then converted to immature osteoblasts via activation of Runx2, β-catenin, and Dix3/5/6 transcription factors. Further, the activation of Osterix leads to the formation of mature osteoblasts. After the completion of the formation of mature osteoblasts, some of these cells flatten and cover the bone surfaces; these are known as bone lining cells. The influence of PTH activates these bone lining cells to produce matrix, and they are also connected with osteocytes through gap junctions.

It has been suggested that bone lining cells function in the bone remodeling process by withdrawing from the bone surface and crafting a canopy over the osteoblasts and osteoclasts. It has been estimated that 60-80% of the osteoblasts die by apoptosis (Bellido et al., 2014; de Gorter, 2013) and remaining osteoblasts flatten to cover quiescent bone surface and the remaining of osteoblasts die by apoptosis. Therefore, the signaling pathway that occurs during osteoblast differentiation is dependent on the types of molecules that ultimately induce bone-promoting cytokines in the mesenchymal osteoblast lineage.

Osteoclasts are the exclusive bone resorption cells of the monocyte-macrophage family. Receptor activators of nuclear factor-ƙB ligand (RANKL) and macrophage-colony stimulating factor are the two cytokines responsible for osteoclastogenesis and come from the hematopoietic lineage. During the bone remodeling process, the precursors of osteoclasts are retracted to the bone surface and then undergo proliferation and differentiation into mature multinucleate cells. The adherence of osteoclasts to the

bone surface via integrin generates the sealing zone, where secretion of hydrochloric acid and acidic proteases occurs. The combined actions of vacuolar H+ and ATPase causes acid production, which further couple with the actions of chloride channels and chloride-bicarbonate exchangers. The activation of these channels targets acidifying vesicles, which fuse with the plasma membrane, causing bone resorption. Lysosomal enzymes and metalloproteinases are also secreted during the bone resorption process, which further degrades hydroxyapatite and matrix proteins. The sealing zone protects the bone surface by providing a tight seal around the ruffle border, which minimizes leakage of the resorbed products. Once the resorption process is completed, osteoclasts undergo apoptosis and osteoblasts take over the resorbed area, leaving the osteoid tissue to undergo calcification (Salo, 1997; Stenbeck, 2002; Teitelbaum, 2003). The remodeling process generally requires 3-6 months, with a distinct sequence of activation, resorption, and formation. Each sequence involves activation, (8 days), resorption (34 days), and formation (120 days or more). The remodeling process occurs on the endosteal surface. These processes occur over the entire bone in small sections of remodeling packets.

Osteocytes are mature stellate-shaped cells and make up over 90-95% of all bone cells. The bodies of osteocytes are encapsulated in the lacunae and extend their cytoplasmic dendritic processes within the mineralized matrix. Quantitative analyses have revealed several cytoplasmic projections that emerge from osteocyte cell bodies. These projections can communicate with each other through the gap junctions within the canaliculi. These gap junctions are formed by the proteins known as connexins.

Accumulating evidence suggests that connexins can function through the hemichannels, which are important precursors for anti-apoptotic activity (Bonewald, 2011).

Osteocytes in contact with the surface of the osteoblasts respond to tmechanical loading via a lacuna-canalicular network. It has been reported that cyclic mechanical loading of osteocytes is responsible for the flow of bone fluid by extravascular pressure (Klein-Nulen, 2013). Besides mechanosensory function, osteocytes regulate phosphate homeostasis, reflecting their role in endocrine function and recently it has been reported that the use of glucocorticoids leads to the apoptosis of osteocytes (Bonewald, 2011). The apoptosis of osteocytes reduces to sense microdamage or repair signals (Bonewald, 2011).

Mechanotransduction

The human skeleton is composed of amazing engineering of three types of bone cells: osteoblasts, osteocytes and osteoclasts. These bone cells are regulated via several cellular processes and in the presence of mechanical loading these cellular signals are converted to biological signals, which influence the bone remodeling processes. Osteocytes play an important role in mechanotransduction, an important mechanism to control bone homeostasis (Klein-Nulen, 2013; Spyropoulou et al., 2015) and any alterations in this process can increase the risk of bone diseases (Cox, 2011).

The exact mechanism of sensing the mechanical loads by osteocytes is still not completely understood. However, accruing evidence supports that mechanical loading causes flow of interstitial fluid within the osteocyte and its dendritic processes stimulate the signaling molecules that regulate the process of bone formation and resorption (Huiskes et al., 2000). The various mechanical signals such as tissue strain, hydrostatic

pressure, and streaming potentials can activate osteocytes. Further, *in vitro* and *in vivo* studies have suggested that in the absence of mechanical loading osteocytes produce factors that stimulate the osteoclasts and that the presence of mechanical loading stimulates upregulation of Wnt/β-catenin target genes and down regulation of sclerostin (Santos et al., 2009; Galea, 2011). Bell et al. (2008) has proposed that the primary cilium of osteocytes is able to sense the hydrostatic pressure generated from the mechanical loading, but no *in vitro* research has been done to test this hypothesis. Therefore, the exact mechanisms by which the cellular components or the sense complexes that are situated in the osteocytes contribute to mechanotransduction remain unclear. Connexins are considered to be important in communicating among osteocytes, especially connexin 43; a lack of connexin 43 increased osteocyte apoptosis with increased osteoclastogenesis (Zhang et al., 2011). Therefore, there is still a lack of evidence regarding the exact structural features that activate osteocytes to function as mechanotransducers.

The knowledge of what causes osteocytes to respond to mechanical stimulation suggests that various mechanical signals are responsible for evoking this process. The production of nitic oxide, prostaglandins, Wnt signaling, bone morphogenetic proteins, and integrin signaling can bridge the gap between mechanical loading and biological responses (Gardinier et al., 2010). The discovery of the Wnt signaling pathway has added to the repertoire of molecules mediating mechanotransduction in bone. Wnt signaling is an important modulator of the bone formation process and can be activated by β-catenin pathways via kinases or GTPases (Kamel, 2010). It has been reported that mechanical loading influences MC3T3-E1, an osteoblast precursor derived from mouse

calvaria, to increase Wnt gene expression. This finding explains how pulsating fluid-flow upregulates mRNA expression of the β-catenin pathway in the signaling cascade and indicates the importance of Wnt signaling in osteocyte biology (Cui et al., 2011).

The expression of sclerostin is found to be higher in mature osteocytes, where it is transported to the bone surface via canaliculi and inhibits bone formation. Studies in animal models have suggested that sclerostin can bind with Wnt co-receptor Lrp5 and inhibit bone formation, where unloading increases SOST expression in the tibia (Robling, 2006). The accumulating evidence suggests that signaling molecules play an important role in orchestrating the recruitment of osteoblasts and osteoclasts, which ultimately results in the adaptation of bone mass and structure (Figure 1).

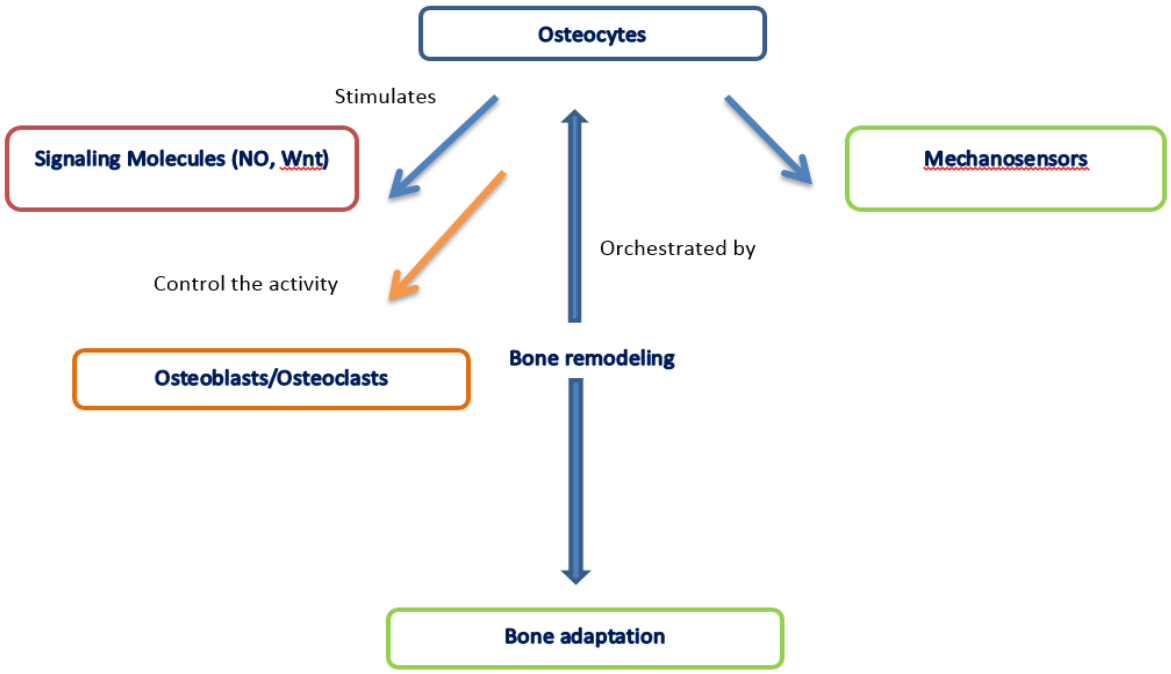


Figure 1. Schematic Diagram Showing Role of Osteocytes in the Bone Remodeling Process. NO, nitric oxide.

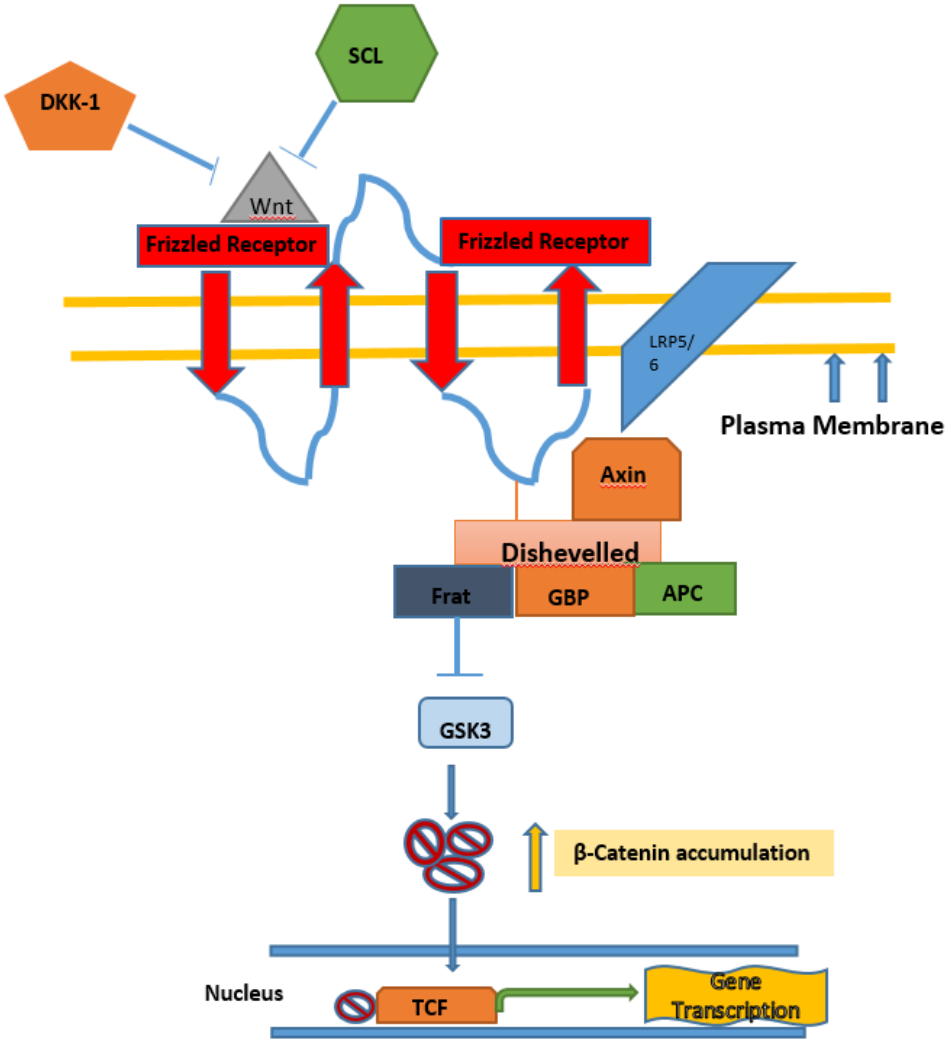


Figure 2. Schematic Diagram of Wnt signaling Pathway

Wnt proteins are important secreted proteins that regulate development, differentiation, the function of various cells and tissues in living organisms. The signaling of Wnt proteins occurs through several pathways, but the Wnt/β-catenin, or canonical, pathway is the most important for understanding bone biology (Westendorf, 2004). The binding of Wnt/β-catenin to the 7-transmembrane domain Frizzled receptor and low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6) coreceptors causes its activation (Moon, 2004). The activation leads to the stimulation of axin and frat proteins, which inhibit the activity of glycogen synthase kinase (GSK3) and hypophosphorylated β-catenin. The formation of stabilized β-catenin then accumulates and translocates in the nucleus, which enhances the transcriptional coactivator to interact with T cell factor and lymphoid enhancer binding factor (TCF/LEF) for Wnt gene transcription (Hay, 2005; and Logan, 2004) as shown in Figure 2.

Wnt interacts with its receptors through canonical and non-canonical signaling. Canonical and non-canonical signaling are important for skeletal homeostasis; however, only canonical signaling function with the activation β-catenin Wnt signaling is a tightly regulated pathway and is inhibited by Wnt inhibitory factors such as sclerostin and DKK-1. LRP5/6 coreceptor activity is also inhibited by these factors. Interaction with this antagonist regulator will cause degradation of β-catenin before it translocates into the nucleus and thus suppresses gene transcription (Mao et al., 2002).

Both LRP5 and LRP6 are essential for the development of normal postnatal bone and it has been reported that LRP5 activates bone formation whereas LRP6 stimulates both formation and resorption (Riddle et al., 2013). LRP6 knockout mice

showed a change in osteoclast activity with a significant reduction in osteoblast number compared to wild-type mice (Li et al., 2013). The fluorescence resonance energy transfer technique was used to show that LRP6 plays an important role in PTH signaling but no evidence in activating Wnt pathway through mechanical loading compared to the crucial role of LRP5 in mechanotransduction (Wan et al., 2008; and Sawakami et al., 2006). This evidence is important to determine the role of LRP5/6 for Wnt and hormone signaling in regulating bone homeostasis.

Additionally, β-catenin is responsible for the differentiation of osteoblasts, stimulating preosteoblast replication and inhibiting osteoblast apoptosis through a different mechanism in postnatal bone growth (Kato et al., 2002; Reya et al., 2005). Mice deficient in β-catenin expression exhibit progression of bone loss, demonstrating the importance of β-catenin in bone biology (Kramer et al., 2010). Therefore, alterations in the expression of β-catenin directly influence the Wnt pathway. The mechanisms of how the Wnt signaling pathway regulates bone mass suggest the generation of osteoblasts from the differentiation of mesenchymal stem cells in the osteoblast lineage together with the suppression of the chondrogenic and adipogenic lineages. Wnt signaling reduces apoptosis of that mature osteoblast and increases the expansion of osteoprogenitor cells via the activation of β-catenin, suggesting the important role or Wnt signaling in bone formation (Almeida et al., 2005; Modder et al., 2011). Deletion of Wnt/β-catenin signaling pathway increases osteoclast number and reduces the bone forming cells (Wei et al., 2011).

Growing evidence suggests that age-related decline in bone mass often results in osteoporosis and the attenuation of Wnt/β-catenin is associated with the loss of bone

mass. Also, aging is associated with an increase in oxidative stress that causes β-catenin in the osteoblast progenitors to follow forkhead family of transcription factors (FoxOs) pathway rather than Wnt. This FoxO pathway leads to attenuation of β-catenin activity, which further decreases the formation of osteoblasts. Therefore, this diversion usually contributes to the age-related changes in the bone mass and increased osteoporosis (Van der Host, 2007; Salih, 2008; Manolagas, 2007; Alemeida, 2007; Lopez-Otin, 2013).

Sclerostin

The Wnt/β-catenin signaling pathway is fine-tuned by secreted glycoproteins that act antagonistically with the signaling pathway. Sclerostin and DKK-1 are recognized as the most potent negative regulators of the Wnt/β-catenin signaling pathway. Sclerostin is the product of the *SOST* gene and is the member of CCN protein family that interacts with LRP5/6 and inhibits the Wnt pathway. Defects in sclerostin have been associated with increased bone mass; in humans, this is known as Van Buchem disease. However, the overexpression of *SOST* alleles is associated with loss of bone mass (Agholme et al., 2011). Knockout of the *SOST* gene in mice has also revealed increased trabecular and periosteal bone mass (Li et al., 2009). Recent findings suggest that sclerostin is highly expressed in osteocytes and its expression is decreased in the presence of mechanical loading (Robling et al., 2006).

Current therapies for the treatment of bone loss are exclusively based on the antiresorptive approach; therefore the development of an antisclerostin neutralizing antibody could provide novel treatment for osteoporosis. During the process of bone remodeling, the secretion of sclerostin by osteocytes stimulates the negative feedback mechanism and prevents BMU overfilling. The modeling process is carried out by

preventing the activation of osteoblasts so that bone lining cells are devoid of osteoblasts. The identification of gain-of-function and loss-of-function mutations suggests that sclerostin bind with LRP5/6 and inhibit the canonical pathway. Recombinant human PTH is considered to be a possible regulator of *SOST* expression; therefore, it is used in the treatment of osteoporosis.

The exact mechanism is still not clearly understood, but it has been reported that the increased sclerostin expression after the application of glucocorticoids can be fine-tuned by PTH and mechanical loading (Yao et al., 2008). Gaudio et al. (2010) compared sclerostin levels in postmenopausal women who cannot mobilize due to stroke to levels in age-matched control subjects. This study found that serum sclerostin was higher in the immobilized subjects compared to the controls, with no change in DKK-1 levels suggesting a potent role of the SOST in addressing strain-induced bone changes.

DKK-1

DKK genes are comprised of conserved members (1-4) that encode secreted glycoproteins with a cysteine-rich domain. The conserved member DKK-3 is not able to inhibit the Wnt signaling pathway. However, DKK (1, 2 and 4) can interact with LRP5/6 in the Wnt signaling pathway. The cysteine domain-2 of DKK-1 gene shows high-affinity binding with LRP5/6 which is enough to inhibit the Wnt signaling pathway (Mao et al., 2002). Reducing the DKK-1 expression in mice resulted in the high bone mass and increasing its expression resulted in osteopenia. These results lead one to surmise that DKK-1 plays an important role in bone formation and can be a target for pharmacological intervention in the treatment of bone diseases (Morvan et al., 2006; Li et al., 2006; Roodman, 2006).

Bone mass homeostasis occurs from the mesenchymal stem cell line which undergoes the signaling pathway that leads to the formation of osteoblasts. It has been reported that DKK-1 inhibits osteoblastogenesis by disrupting the osteoblast differentiation pathway, which prevents endochondral osteoblasts formation (Chen et al., 2007). It has been well documented that osteoprotegerin and RANKL are responsible for the bone formation and resorption and the ratio of osteoprotegerin and RANKL determines the net effects of resorption (Diarra et al., 2007). Studies in animal models suggest that DKK-1 exerts a negative effect on osteoprotegerin expression that favors the increased resorption activity by shifting the osteoprotegerin and RANKL ratio (Diarra et al., 2007). Also, mechanical loading decreases the expression of sclerostin and DKK-1, supporting the importance of mechanical loading in the pathogenesis of disuse bone loss (Gaudio et al., 2010).

Mechanical Loading

Mechanical loading and bone adaptation is a complex process and various models have been utilized to gain insight into this adaptive process. Also, mechanical loading is an integral part of the development and growth of a weight bearing skeleton (Turner et al., 2009). As proposed in the Utah Paradigm of Skeletal Physiology, bone formation and resorption occur when the mechanical strains are higher and lower than the strain threshold (Frost, 1999). When modeling is turned “ON”, a strain threshold is surpassed and when it is turned “OFF, a strain remain below its threshold. Similarly, the term "remodeling" is used to signify strains below the modeling threshold, when modeling is OFF. Bone remodeling involves several threshold ranges such as “conservation mode” and “disuse mode.” When bone formation and resorption are

equivalent, strains just below the threshold are the "conservation mode." When bone resorption exceeds formation, leading to loss of bone mass, and strains are below the conservation mode, the bone is considered to be in "disuse mode". Prolonged reduction of strain in disuse ensues the condition known as "disuse-pattern-osteopenia," which can be acute or chronic and can be characterized by normal bone but larger marrow cavities. Further, Frost (1999) explained that the modeling and remodeling processes are driven by drifts and basic multicellular units (BMUs).

Muscle also plays an important role in postnatal bone strength and mass. The voluntary loads and strains can be influenced by muscle contraction. As a person ages, the pattern of muscle strength increases and then plateaus, and after 30-40 years, a gradual loss of muscle strength takes place that is similar to loss of bone mass. Compared to muscle strength, the bone formation and resorption process is slower and takes time for adapting to the usual strain that is placed on it. Thus, mechanical loading provides a positive influence on the skeletal strength that can withstand the load and prevent a fracture that could be due to age-related bone loss, disuse, or microgravity environment (Price et al., 2011). Further, that bone loss can be reduced by increased physical activity is a local phenomenon for the adaptive response and can be locally controlled (Sugiyama et al., 2010).

However, the mechanostat theory described above is not able to explain how bone cells stimulate the processes of bone formation and resorption. Animal model study has explained the extrinsic mechanisms for mechanical loading where bone adaptation is dependent on the frequency and the magnitude of the strain the bone experiences (Meakin et al., 2014). These mechanisms indicate that static load of zero

frequency would not influence bone adaptation process. Further, based on Turner's rules, adaptation can be acquired either through increased strain magnitude and decreased frequency or decreased strain magnitude and increased frequency. Similarly, the concept of diminishing returns after prolonged exercise in rats suggests that the anabolic response showed no benefit on bone after increasing the loading cycle or duration (Umemura et al., 1997). Also, adding a 14-second rest period between the loading cycles can improve bone formation and suggests that rest is necessary for the osteogenic stimulus (Robling et al., 2001).

In addition, various types of exercise such as treadmill running, jumping, and swimming in animal models have helped researchers to understand that the mechanostat theory (Yeh et al., 1993; Bourrin et al., 1992; Ju et al., 2012), which can be applied in human studies. The positive relationship between bone mass and exercise has encouraged researchers to recommend that individuals engage in a physical activity so that the risk of osteoporotic fracture can be reduced. Given that prolonged exercise duration can influence the bone cells to ignore mechanical loading and the development of an osteogenic index (OI), explain the effectiveness of exercise protocols on bone mass (Turner and Robling, 2003). The utilization of OI by several studies reported that OI activities decline with age. As a predictor of bone health, the OI uses ground reaction forces, duration, and frequency of participating in a given physical activity (Nilsson et al., 2009).

Bone Responses to Physical Activity in Premenopausal Women

Frost's Utah paradigm of skeletal physiology has contributed insight that there is a threshold above which bone must be stressed in order to be adapted. With the various

animal and human studies it has now been clearly established that physical activity has provided a positive influence on skeletal mass.

With this perspective from Turner's three rules for bone remodeling, loads must be dynamic, non-routine, and short duration; mechanical strains should have progression of overload because bone cells will adapt and will not respond to the customary loading. The time frame of a complete bone loading requires 3-6 months and the training studies are required to detect the changes in the bone parameters via invasive and non-invasive techniques. Therefore, many studies have quantified the influence of physical activity in bone health through longitudinal studies, cross-sectional studies and through meta-analyses of different populations. Age also plays an important to a role in determining the effect of physical activity on skeletal health. The age at which an individual participates in high impact exercise and if the activity level is continually maintained throughout the life are some of the major determinants of the influence of physical activity on bone health. One study examined the association of physical activity and bone mass in healthy women and found that physically active young women with high calcium intake had higher bone mass in distal tibia trabecular bone compared to physically active postmenopausal women. The results suggest that mechanical loading in young age is important for long-term conservation of mechanical loading (Uusi-Rasi et al., 1998).

In a 28 year follow-up study, the habitual childhood and adolescent physical activity or inactivity on tibial pQCT bone at the age of 3-18 years was assessed. Further, at the age of 31-46 years, assessment of cortical and trabecular bone density was performed. The results suggest that childhood physical activity was associated with

increased cortical area of the tibia, BMC, and SSI in premenopausal women. This led the researchers to conclude that that frequent habitual physical activity in adolescence provides a positive influence on tibial bone size and geometry as individuals age (Tolonen et al., 2015).

Sherk et al. (2010) conducted a study to compare bone mineral density and bone quality in adult rock climbers, resistance-trained men, and control subjects. The results showed that resistance-trained young men had a significantly higher lumbar spine and femoral neck BMD compared to rock climbers and control men. However, no significant differences were observed for the cortical bone variables. The results suggest that bone adaptations among climbers could have been restricted to some specific sites such as the fingers, which could experience loads above modeling threshold.

One cross-sectional study reported the effect of past sports activity on bone density parameters in both men and women (Kato et al., 2014). Both men and women of 20-23 years participated in the study, where MRI and DXA were used for the body composition and bone density assessments, respectively. Women participating in sports activity in elementary school had significantly higher BMC, total proximal femur, and muscle-cross-sectional area compared to women in the no sports group. Also, OI was found to be correlated with MRI measures of bone geometry, whereas DXA variables were considered as effective indicators of past activity both in men and women. The results of this study suggest that participating in high impact exercises before and early puberty showed enhanced femoral mid-diaphyseal bone geometry (Kato et al., 2014).

In a cross-sectional study, the effects of physical activity on cortical bone in jumpers, swimmers, and untrained control groups was studied using pQCT. It has been

well established that loading and frequency play important roles in bone adaptation that can then further influence the geometric properties of bone. The results on tibia analyses showed that polar moment of inertia and SSI, area and thickness were significantly higher in jumpers compared to swimmers and the control group. Similarly, female jumpers had significantly higher cortical BMC, SSI, thickness, and area but lower vBMD, suggesting that jumpers could have strong bone to overcome of the compressive and bending strains. Similarly, in swimmers, the enlarged endocortical area could be due to the early training and influence from the endocrine system. Therefore, long-term participation in physical activity by athletes is associated with increased cortical geometric adaptations rather than vBMD improvements (Liu et al., 2003).

Jumping mechanography is an important tool in the assessment of muscle function and peak strains, and the peak power can be calculated as the product of force and velocity via countermovement jumps. It has been now well documented that peak power represents the activity of hip, thigh and calf muscles, whereas peak force reflects Achilles tendon elasticity (Anliker et al., 2011; Veilleux, 2010). Heinonen et al. (2001) conducted a cross-sectional study to investigate the influence of extreme impact loading on bone parameters with the DXA and pQCT in 8 triple jumpers and controls. The results showed that femoral neck and lumbar spine were 31% higher in jumpers compared to controls. Similarly, distal femur and BSI measurements were 4-6 and 19-31% higher among jumpers compared to the control group. In jumpers, the loading effect was observed higher in the cortices after an increase in the trabecular densities.

Another cross-sectional study compared bone mass and geometry among long and middle distance runners, race-walkers, sprinters and sedentary control participants (Wilks et al., 2009). Female sprinters and middle distance runners had 15% and 12% larger cross-sectional polar movement of resistance and higher trabecular vBMD than control. Surprisingly, cortical vBMD was highest in the controls, while cortical BMC, area, and strength were higher in the sprinters. The results suggest that running speed is positively associated with geometric tibia measures whereas cortical vBMD is negatively associated with loading frequency (Wilks et al., 2009). Further, a meta-analysis study on exercise and BMD in premenopausal women (7 studies, n=466, g= 0.342 for femoral neck; 6 studies, n=402, g=0.201 for lumbar spine) reported that participating in exercise for more than 24 weeks significantly increased femoral neck and lumbar spine BMD. In this study, standardized effect sizes were calculated for each result and pooled using random-effect models (Kelley et al., 2013).

A study of short-term jump activity on bone metabolism by Kishimoto et al. (2012) reported that two weeks of jump activity consisting of 10 jumps/day significantly decreased bone resorption markers in 26 non-athlete females of 19-24 years of age. Further, this study also revealed a significant decrease in the early marker of bone formation, bone alkaline phosphatase, and no change in the late marker osteocalcin. The results suggest that short training protocols can attenuate the bone turnover markers and controling the time intervals, diet, and use of contraceptives can influence the bone formation markers.

Similarly, a study by Baxter-Jones et al. (2008) reported the relationship between physical activity and bone mass from young to adult phase both in men and

women. Subjects were categorized into active, average and inactive groups and the results showed that active adolescent females had 9 % and 10% more adjusted BMC, total hip and femoral neck compared to the inactive group. The researchers surmised that participating in physical activity conserved the skeletal benefits throughout the adult phase.

In the assessment of jumping mechanography on hip BMD, Tucker et al. (2015) used a longitudinal study to determine the effect of two jumping programs in premenopausal women. Sixty premenopausal women ages 25-50 years participated in the study and were categorized into three groups of jump 10, jump 20, and control. DXA was used for the hip BMD assessment during baseline, at 8 weeks, and at 16 weeks. Subjects were instructed to jump on a force plate at 30 secs intervals for 10 jumps for jump 10 group and 20 jumps for jump 20 group. Control subjects were instructed to perform stretch for a total of 10 minutes for 6 days. The unadjusted percent change in hip aBMD at 8 weeks was significantly greater in the jump 20 group compared to the control and there was only a marginal increase in the jump 10 group. Similarly, at 16 weeks after adjusting for confounding variables, hip aBMD was significantly higher ($p=0.009$). These results suggest that high impact exercise, such as jumping 20-30 times daily, can improve the skeletal health and prevent fracture risk in the future.

In a randomized, controlled trial study by Bailey and Brooke-Wavell (2010) categorized premenopausal women of 18 -45 years into exercise and control groups. The exercise group was further categorized into 0, 2, 4 or 7 days/week for 6 months with sets of 10 hops on one limb followed by 15 seconds of walking. This study aimed

to investigate the effectiveness of high-impact exercise on BMD. DXA was used for the body composition and bone density assessment. The results showed significantly increased peak ground reaction forces and 2% in femoral neck BMD in the exercise group compared to the control group, suggesting the positive influence of high-impact exercise on reducing hip fragility.

Liang et al. (2011) conducted a randomized control trial to evaluate the effect of high impact exercise in premenopausal women between 20-35 years. In this study, subjects were randomly assigned to high impact aerobic exercise, moderate intensity strength training, or a control group. Whole body and heel and wrist aBMD were measured using DXA. After a 12-month intervention, there was an increase in heel aBMD (4.4%) and leg press strength compared to the baseline, but no significant increase in aBMD was observed. Most of the untrained women were at their peak aBMD level and small sample size could have attributed the lack of statistically significant findings.

Similarly, Warren et al. (2008) conducted a longitudinal study in premenopausal women of ages ranging from 25-44 years old. This study included 148 sedentary premenopausal women categorized into strength treatment and control group. Subjects' bone density parameters were measured at baseline, 12 months and 24 months. DXA was used for the assessment of body composition, bone mineral content, aBMD, and bone area of the proximal femur and lumbar spine. Leg press and bench press were used for the muscle strength assessment, with these measurements separated by two weeks. Physical activity assessment was performed by accelerometer where subjects were instructed to wear the device for 4 days. There was a significant difference between the

two groups for femoral neck BMC, with 1.5% decrease in the control group suggesting the importance of mechanical loading in bone health.

Sclerostin and DKK-1 Responses to Physical Activity

Signaling pathways allow a bone to adapt depending on its biomechanical requirements. In animal models, it has been shown that mechanical loading decreases sclerostin production and unloading increases SOST production. Gaudio et al. (2010) conducted a cross-sectional study in 40 elderly women who were immobilized for 6 months after stroke and 40 control subjects to determine the effect of unloading on bone. Bone turnover markers, sclerostin, and DKK-1 were measured from serum samples. The results revealed significantly higher bone resorption markers and sclerostin levels in immobilized women compared to controls. However, no significant differences were observed for DKK-1 between the groups. The results suggest that disuse or lack of mechanical loading could accelerate the increase in those markers, which could result in the disuse pattern of osteoporosis.

In a cross-sectional study, Mirza et al. (2010) compared pre- and postmenopausal women and correlated sclerostin with free estrogen, bone markers, and parathyroid hormone levels. In this study, the average age of premenopausal and post-menopausal women was 26.8 and 56.8 years, respectively. The results showed that serum sclerostin levels were significantly higher in postmenopausal women compared to premenopausal women. Also, negative correlations were observed between levels of sclerostin and both free estrogen and parathyroid hormone in postmenopausal women suggesting that estrogen deficiency causes secretion of more sclerostin in the circulation and might regulate bone by acting as an endocrine hormone. Further, this study included

contraceptive users with variety of sex hormones, which make difficulty in assessing the total estrogen in premenopausal women. A cross-sectional study by Garnero et al. (2013) that reported parathyroid hormone levels were negatively associated with sclerostin levels, but different in that no significant association with estradiol levels was observed.

A cross-sectional study by Amrein et al. (2012) investigated sclerostin's association with age, gender, bone variables, and levels of physical activity. This study included 161 healthy men and 34 premenopausal women who had their sclerostin, BMD, biochemical parameters and physical activity levels evaluated. The results showed that men had significantly higher unadjusted sclerostin levels compared to premenopausal women and all the premenopausal women were younger than male subjects. No significant correlation was observed between sclerostin and the measured biochemical parameters. However, adjusting for confounding factors such as skeletal size (men had 21% higher skeletal mass), age and physical activity levels, sclerostin levels did not differ significantly between the genders.. Overall, in healthy adults, most physically active individuals had significantly lower sclerostin levels than less active individuals. However, this conclusion cannot be applied to premenopausal women, as the sample size was comparatively lower than for adult men and the use oral contraceptive was not reported.

Ardawi et al. (2011) studied the determinants of serum sclerostin in healthy pre- and postmenopausal women between the ages 20-79 years old. This study also aimed to establish the normative reference values for sclerostin, age-related changes and effect of menopause. A total of 1,803 women participated in the study and the premenopausal age range was 20-45 years old. The results showed that postmenopausal women had

significantly higher levels of osteocalcin and some bone resorption markers compared to premenopausal women. Also, postmenopausal women showed higher sclerostin levels. The regression analyses showed that sclerostin increased linearly with age, with a steep increase until age 35, remained stable between 35-45 years and increased sharply again after that 45, suggesting the importance of sclerostin with aging.However, from the exercise perspective, this study lacks an explanation about physical activity levels in both pre- and postmenopausal women to address the relationship between mechanical loading and sclerostin levels.

Modder et al. (2010) conducted a cross-sectional study to determine the relationship of age, gender and bone mass with sclerostin levels in men and women. This study included 318 men and 362 women, both pre- and postmenopausal. The results of this population-based study showed that men have significantly higher sclerostin levels than women (33.3 ± 10. pmol/L versus 23.7 ± 0.6 pmol/L). Sclerostin levels were significantly higher with aging in both men and women. The authors noted that the higher sclerostin levels in men could be due to their greater skeletal mass that produces more circulating sclerostin. The specific dose response between sclerostin and inhibition of bone formation is still unclear, which necessitate to determine the dose-response relationship between bone formation and bone markers.

Ardawi et al. (2012) conducted a cross-sectional study with a subgroup followed up longitudinally to determine the physical activity response of sclerostin levels in premenopausal women. In this study, 58 women were followed longitudinally during an 8-week course of physical activity training (4d/wk) and compared with 62 control subjects. The exercise protocol included 120-min sessions for 4 d/wk, with 20 min

walking at increased speed and an endurance physical activity of 25 min running, walking on the treadmill for 20 min, cycling for 10 min, and step-ups for 10 min. These exercises were followed by flexion, extension and mobility exercises for upper and lower body for 35 min. After the completion of 8 weeks training, women participating in the physical activity training for 4d/wk had significantly lower sclerostin levels compared to the sedentary individuals. Following the 8 weeks intervention, sclerostin levels were significantly lower by 33.9% but significantly higher bone formation markers in the physically active training group, suggesting the importance of mechanical loading in skeleton biology.

Butler et al. (2011) conducted a study correlating the expression of DKK-1 with BMD. In this cross-sectional experimental cohort study, 18 patients with low BMD and 18 with normal BMD (23 female and 13 male) participated. DXA was used for the BMD analysis of lumbar and femoral sites. DKK-1 levels were significantly higher in the osteoporosis group compared to control ($p=0.001$). Further, serum DKK-1 levels were inversely associated with femur t-score, lumbar z-score, and femur z-score ($p<0.05$). The results suggest that expression of DKK-1 is a pivotal aspect in the assessment of osteoporosis. However, in this study, no significant correlation between aging and DKK-1 was observed. Also, this paper did not report the physical activity levels of the subjects.

A cross-sectional study on ultramarathon runners has shown a significant decrease in DKK-1 levels after the marathon but no changes in sclerostin levels (Kerschan-Schindi et al., 2015). In this study, 19 participants had venous blood samples drawn before, immediately after and 3 days after the start of the race. The researchers

also found bone formation marker significantly decreased after the race and showed a trend of increase thereafter. The bone resorption marker (CTX) was significantly increased after the race and showed a trend of decreasing thereafter. The results suggest that exercise is associated with low-level inhibition of Wnt signaling inhibitors, thereby increasing the differentiation of bone-forming cells. Therefore, short-term participation in an ultradistance race influences the uncoupling of bone turnover but endurance exercise also seems to initiate a long-term positive influence on skeletal mass.

To study the disuse-induced bone loss, Frings-Meuthen (2013) conducted a cross-sectional study to determine sclerostin and DKK-1 levels in the bed-rest condition in young males. One group participated in 14 days head down tilt bed rest and the other group participated in 21 days bed rest, respectively. In both groups, sclerostin levels increased during the bed rest and declined at the end of 14 and 21 days. In study 1, DKK-1 decreased from baseline level until the recovery period, whereas the 21-day study showed increased DKK-1 levels and decreased 3 days after the end of bed rest. The 14-day bed rest group did short walks that could have caused the decrease in the DKK-1 levels. The results suggest that DKK-1 reacted faster to unloading compared to sclerostin as DKK-1 could have higher affinity to LRP5/6, which trigger osteocytes to increase DKK-1 levels. However, small sample size could have affected the results, but the concluding point was that disuse can alter the Wnt signaling pathway by increasing sclerostin and DKK-1.

One randomized study reported the levels of serum sclerostin in 107 female sedentary obese older subjects in response to the exercise training. Subjects were categorized into control, diet, exercise, and diet-exercise group. The exercise groups

were instructed to perform aerobic, resistance training, and balance exercises for 1 year. Bone density and sclerostin measurements were collected at baseline, 6 months and 12 months. No significant changes in sclerostin levels were observed from baseline to 12 months except for the diet group, where a significant increase was observed at 12 months ($p<0.05$). Further, hip geometry analysis showed deteriorating results in the cortical thickness, area and femoral shaft for the diet group. However, no significant changes were observed for the exercise-diet group. The results suggest that exercise training improved BMD and bone geometry and, when added to the diet, it may inhibit the weight loss-induced increase in sclerostin. This could further attenuate bone loss and preserve bone geometry (Armamento-Villareal et al., 2012).

Mosti et al. (2014) conducted a randomized controlled trial in the assessment of BMD with maximal strength training. This study included 30 healthy women of 18-27 years who were categorized into a training group and control group. The training group performed 12 weeks of squat maximal strength training with 85-90% 1RM focusing on progressive loading with high acceleration in concentric phase. Both groups had baseline and post-test measurement of physical capacity parameters, anthropometric parameters, BMD, and serum markers. The dynamic rate of force development and peak force and 1RM were significantly higher in the training group compared to the control group. Similarly, BMD and BMC at the lumbar spine increased by 2.2% and 3.4% respectively, in the training group ($p<0.05$). No significant changes occurred in the levels of sclerostin and bone resorption markers. This paper indicated that low sample size and duration of the training could have altered the results. These results were consistent with the study

conducted by Kovarova et al. (2015), where no significant changes were observed for sclerostin levels following the resistance exercise.

Summary

Accumulating evidence suggests that mechanical loading is the crucial modulator for bone metabolism. Animal models have shown that disuse or unloading patterns can cause reduced bone formation, which can be connected with human studies exploring issues such as sedentary behavior, bed rest, and paralytic condition. Conversely, participating in high-impact loading is associated with active bone formation. The exact involvement of physical and biological mechanisms in bone adaptation is still not clear, but some biological key players to control mechanotransduction have been identified. These players include the flow of interstitial fluid within the osteocytes, which stimulates the signaling molecules that modulate the bone formation and resorption process. The activation of osteocytes occurs by various mechanical signals such as tissue strain, pressure, and other streaming potentials. Substantial evidence suggests that signaling pathways play important roles in regulating the process of bone metabolism. The Wnt signaling pathway transduces the mechanical signals and regulates bone metabolism. However, the unloading pattern increases the release of sclerostin and DKK-1 at the endosteal surface of the long bones. Therefore, better insight into the complex association between physical activity and signaling markers such as sclerostin and DKK-1 will provide an early approach for identifying fracture risk in osteoporosis.

The increase in BMD during early puberty is followed by a slower increase in BMD and consolidates the mineral during the early thirties until the peak bone mass is

achieved (Clarke and Khosla, 2010). Therefore, maintenance of bone mass during the premenopausal phase is important as age progresses. To conserve bone mass, it is necessary to expose the skeleton to mechanical loading or exercise. Based on the literature, it has been well established that various high-impact exercises can decrease those Wnt signaling inhibitors, facilitating the bone forming cells. Also, epidemiological evidence suggests that physical activity or exposure to high impact exercise can reduce fracture risk and improve muscle force and coordination. Therefore, findings from the literature necessitate framing the type and duration of physical activity that decrease the production of sclerostin and DKK-1, which will then prevent bone formation cells from undergoing apoptosis.

Chapter 3: Methodology

The primary purposes of this study were to: (1) compare serum concentrations of sclerostin and DKK-1 in young (20-30 years) and middle-aged (35-45 years) premenopausal women; (2) evaluate the differences in serum sclerostin and DKK-1 concentrations based on the physical activity status (low, moderate, and HEPA-active). The secondary purposes of this study were to: (1) evaluate relationships of sclerostin and DKK-1 with aBMD and volumetric bone mineral density (vBMD) in young and middle-aged premenopausal women; (2) evaluate relationships of sclerostin and DKK-1 with leg strength; and (3) evaluate relationships of sclerostin and DKK-1 with jump power. Dependent variables included the serum levels of sclerostin and DKK-1; aBMD and BMC of the total body, lumbar spine, and dual femur; vBMD of the trabecular and cortical bone at 4%, 38%, and 66% of the tibia. This chapter describes the methods used for this study, including sample sizes, data collection procedures, and data management and analyses steps.

Participants

Participants for this study were premenopausal women between ages 20-30 years (n=25) and 35-45 years (n=25), and of diverse ethnicity. Participants were recruited from the Norman and Oklahoma City Metropolitan area via mass email, flyers, classroom recruitment, message recruitment, word-of-mouth, and advertisement. Prior to the first visit to the Bone Density Research Laboratory for testing, participants were screened via a screening checklist. All the protocols were approved by the University of Oklahoma Health Sciences Center Institutional Review Board before the start of this study (IRB No. 6945) (Appendix A).

Participants were screened based on the screening checklist questionnaire through email, phone or in person. Participants were excluded from the study if they met any of the exclusion criteria based on their responses to the screening questionnaire (Appendix C). Eligible participants were scheduled for the first visit to the Bone Density Research Laboratory. During the first visit, each participant completed the written informed consent, HIPAA form, health screening questionnaire, calcium intake questionnaire, menstrual history questionnaire, BPAQ, and IPAQ (Appendix B).

The available data on Wnt signaling inhibitors in humans, particularly in young and middle-aged premenopausal women, are sparse. The sclerostin results from a cross-sectional study in pre- and postmenopausal women published by Modder et al. (2011) were used to perform a power analysis using G*Power 3.1 (Faul et al., 2007). The effect size (d) for sclerostin differences between pre- and postmenopausal women was determined to be 1.18, a large effect size (Cohen, 1992). This effect size was used to estimate the required sample size for this study based on a statistical power of 80%. A sample size of 13 for each group was determined to be adequate; however, due to the possibility of dropouts and outliers, a sample size of 25 for each group was used for this study.

Inclusion Criteria

1. Participants were healthy, premenopausal women aged 20-30 years and 35-45 years.
2. Women with normal menstrual cycles (10-14 cycles in 12 months).
3. Body weight was less than 300 lbs (136.3 kg), which is the weight limit for DXA.

4. Height was less than 6 feet in order to get accurate values for both DXA and pQCT.

Exclusion Criteria

1. Women who were pregnant.
2. Women taking medication that can affect bone density or metabolism, such as mineralocorticosteroids, glucocorticoids, bisphosphonates, and calcitonin.
3. Women with an artificial knee, joint, or any other metal implantation in their bodies.
4. Women with recent surgery, fracture, and open wounds.
5. Women who had cardiovascular disease or uncontrolled hypertension.
6. Women currently smoking or had smoked regularly within the past 6 months.
7. Women with physical disabilities that prevent them from performing weight lifting exercises.

Research Design

This study was a non-randomized cross-sectional design with two independent groups: young women, 20-30 years, and middle-aged premenopausal women, 35-45 years. There was no intervention for this study. The purposes of this study were to compare serum concentrations of sclerostin and DKK-1 in young (20-30 years) and middle-aged (35-45 years) premenopausal women. The present study also evaluated the differences in sclerostin and DKK-1 concentrations based on the physical activity status (low, moderate, and HEPA-active). This study further analyzed the relationship between bone density variables, jump trials and leg strength with the serum concentrations of sclerostin and DKK-1. This study required 3-4 visits to the Bone

Density Research Laboratory. During the first visit, each participant was instructed to complete the questionnaire regarding health, menstrual history, BPAQ, IPAQ, dietary calcium intake on a daily basis, physical activity readiness questionnaire (PAR-Q). Participants completed informed consent and HIPAA forms. These questionnaires provided information regarding medical history and exclusion criteria for each woman. These questionnaires asked about age, gender, levels of physical activity levels, lists of medicine taken over the past 6 months, and family history of cardiovascular diseases before the age of 50. During the second visit, a blood sample was drawn at Goddard Health Center to quantify the levels of sclerostin, DKK-1, and follicle-stimulating hormone (FSH). Serum FSH was measured only in the middle-aged women to confirm that they were not perimenopausal.

During the third and fourth visits, both areal and volumetric bone density were assessed using DXA and pQCT. Following this, jump power and leg strength were assessed by using a jump mat and a leg press machine. To assess the test-retest reliability of the bone scans by DXA and pQCT, and the neuromuscular performance tests, a subset of 15 women were asked to return for the fourth visit (procedure were exactly the same as the third visit). We recently installed a new DXA machine in the Bone Density Research Laboratory, therefore we re-established the precision of the BMD variables measurements. Previous precision studies in this laboratory for pQCT and muscle performance variables had been done on young college-aged participants, which ensures that accurate high-quality data are collected across a wide variety of age groups. A complete summary of the research design is shown in Figure 3.

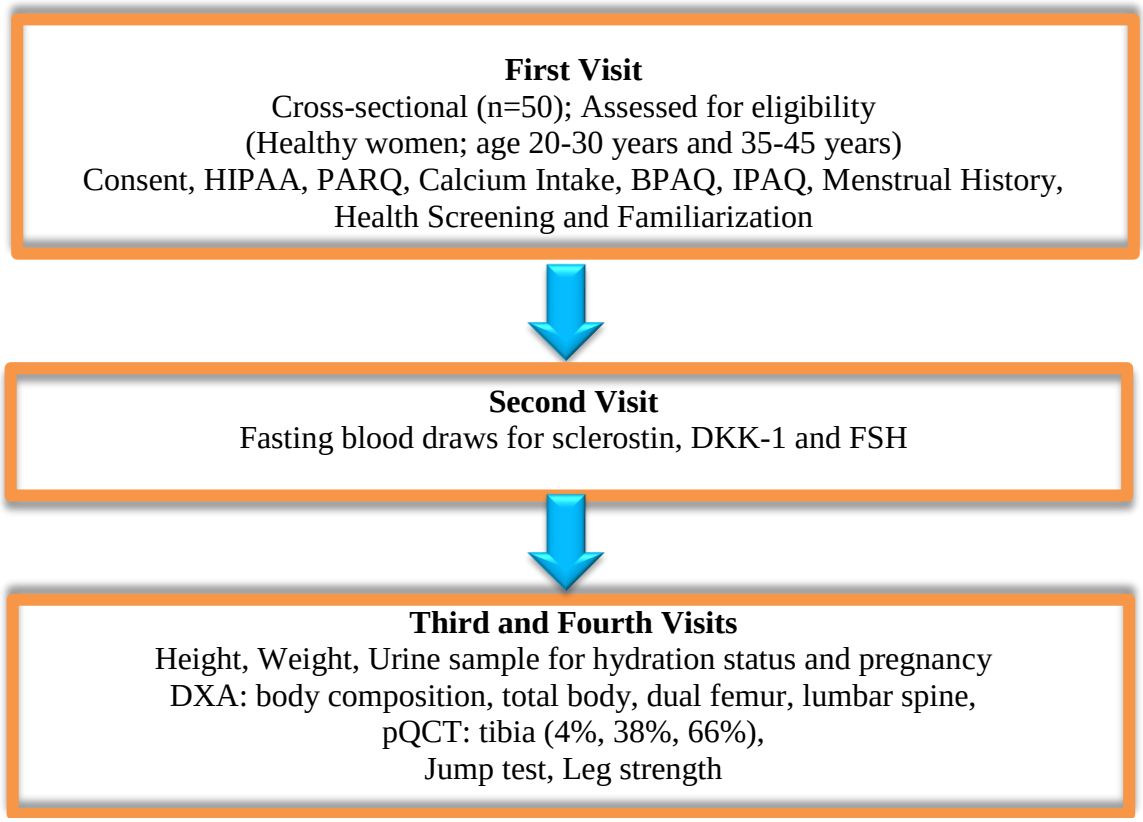


Figure 3. Research Design

Bone-Specific Physical Activity Questionnaire (BPAQ)

This questionnaire evaluated the current and past status of participant's bone-loading physical activities and sports. The questionnaire contained questions about the participant's participation in sports activities within a year and the age at which they started to participate. It has been well established that osteogenic exercise influences bone adaptation by creating stress via muscle contraction (Turner and Robling, 2003). BPAQ was validated with other methods and is a good predictor of BMD, and its loading values were determined by measuring the ground reaction force for specific bone-loading activities (Beck and Weeks, 2008).

International Physical Activity Questionnaire (IPAQ)

This questionnaire contains 27 questions and is designed to yield scores to designate low, moderate and high intensity physical activity. Data for each activity (low, moderate, and high intensity) were converted metabolic equivalent task for 7 days (met-minutes.week^{-1}) (Hagstromer, 2006). IPAQ has been well validated with other methods in assessing the physical activity scores (Craig et al., 2003).

Calcium Intake Questionnaire

This questionnaire consisted of daily calcium intake information based on certain foods that are consumed in a day or a week by participants. The data allowed the researcher to calculate an estimation of the amount of calcium consumed daily based on the content of the specific foods. Participants provided information on any type of calcium supplements they were consuming along with their doses and generic names. The dietary calcium intake questionnaire was derived from a validated and quantitative food frequency questionnaire (Musgrave, 1989).

Menstrual History Questionnaire

A menstrual history questionnaire was used to document participants' menstrual cycle characteristics and hormonal contraceptive information such as dosage, type, and duration of use. Participants provided information on the length of the menstrual cycle, current menstrual status, length of flow, date of the last cycle, premenstrual syndrome, missed a period, and details on past history of taking hormonal contraception. This questionnaire also asked for information on the age at which her regular period started, amenorrhea in menstrual history, and any concern with hormonal abnormalities.

Anthropometric Measurements

Height was measured in centimeters with the individual standing with her back against the wall stadiometer (Novel products Inc., Rockton , IL) without shoes, head facing forward, arm at the side, heels against the wall, and holding her breath. Weight was measured in kilograms with minimal clothing, without shoes, and empty pockets via a digital weight scale (Tanita Corporation of America, Arlington Heights, IL).

Areal Bone Mineral Density (aBMD)

DXA (GE Lunar Prodigy, Version 16, Madison, WI) was used to measure BMC (g) and aBMD (g/cm^2) of the total body, anteroposterior (AP), lumbar spine (L1-L4), and dual proximal femur, which include femoral neck, trochanter, and total hip. Body composition variables such as percent body fat, fat mass, bone free lean body mass and fat free mass were also measured during the total body scan. The DXA scanner uses a filter-equipped X-ray tube, which converts polychromatic X-ray beams to low and high energy peaks. These attenuations are useful to provide the typical scan images. Participants were exposed to 0.05-1.5 mrem of radiation during the DXA scans, which is equivalent to spending one extra day in sunlight per year. Quality assurance testing (QA) was performed each day to ensure the proper operation of DXA. During the QA, a calibration block of known density was scanned to conduct a series of mechanical functioning tests performed by the software. These procedures were performed prior to scanning the first participant each day. The images from DXA are considered as 2D images; therefore, the thickness of the bone cannot be measured by DXA.

Additionally, each participant's hydration status was measured from a urine sample with an optical refractometer (VEE GEE CLX-1, Rose Scientific Ltd., Alberta,

Canada) prior to the bone scans. This ensured hydration levels, as measured by urine specific gravity, were within the normal range of 1.004-1.029. It has been reported that hydration level could influence the accuracy of the body composition measurements. Therefore, participants with hydration status outside the range were rescheduled to return after normal hydration levels were established. A pregnancy test was performed using test strips (SAS Pregnancy Strip, SAS Scientific, San Antonio, TX) prior to the bone scans. The test strip was positioned at the top of the urine sample in a vertical position with arrows pointing downward, and not touching the stop line in the urine sample. The test was read after 4 minutes to check for positive or negative results.

During the DXA scans, participants laid in a supine position on the scanning table after removing their shoes and any metal or image-attenuating materials. For the total body scan, the participant remained in the supine position with hips and shoulders aligned to the center of the DXA table, with knees and ankles secured with Velcro straps. For the AP lumbar spine scans, the legs were supported on a foam block, which ensured a 45-90 degree angle at the hip joint and flattened the spine on the DXA table. Laser crosshairs were placed 3 cm below the umbilicus so that the iliac crest and T12 vertebra were visible. Participants were instructed to cross their arms so that the upper arm was perpendicular to the DXA table. The scan progressed from L5 to T12 vertebrae. The scan speed for the total body and lumbar spine was determined by measuring the thickness at the subject's naval (Thick = > 25 cm; Standard = 13-25 cm; and Thin = < 13 cm). The dual femur scan was performed by placing the feet in the foot brace so that the leg was internally rotated. This ensured the proper exposure of the

femoral neck and the femur in the scanning boundary. The laser crosshairs were placed 7-8 cm below the trochanter so that the ischium was visible.

The software analyzed and performed all the necessary adjustments during scanning, depending upon the regions of interest (ROI) and either separate individual vertebrae or intervertebral markers. The *in vivo* precision and accuracy of the DXA RMS %CV in the Bone Density Research Laboratory is 0.7% for the total body BMD, 1.4% for the spine, and 0.6% for total left and right hip BMD, 0.6% for right trochanter, 0.7% for left trochanter, 0.9% for left femoral neck and 1.01% for left femoral neck. The *in vivo* precision of DXA RMS %CV for body composition variables is 2.0% for percent body fat and fat mass, 1.9% for bone free lean body mass, and 1.7% for fat free mass.

Volumetric Bone Density (vBMD) Measurements

A pQCT XCT 3000 scanner with software version 6.00 (Stratec Medizintechnik GmbH, Pforzheim, Germany) was used to measure cortical vBMD (mg/cm^3), BMC, (mg/mm), area (mm^2); trabecular vBMD (mg/cm^3), BMC (mg/mm), area (mm^2), and total vBMD (mg/cm^3) of 4%, 38%, and 66% of tibia sites. Compressive, bending and torsional strength was estimated as bone strength (BSI), stress-strain indices (SSI) (mm^3), and moment of inertia (IP). The variables that indicate bone size and shape were measured as periosteal (Peri C) (mm) and endosteal circumference (Endo C) (mm).

Calibration of the pQCT was done prior to the actual scan for each test day by a cone phantom. Participants' information was entered into the computer after calibration. Prior to the scanning, participants' tibia length of the non-dominant leg was measured from the tibia plateau to medial malleolus. Partcipants were instructed to place their

non-dominant leg into the gantry and to remain still throughout the scan. The scout view identified the reference point at the medial malleolus. Following this, 3 sets of scanning were performed at 4%, 38%, and 66% of the non-dominant tibia. The *in vivo* precision (RMS %CV) for the tibia ranges from 0.3-1.1% for the total bone variables, 0.7-3% for trabecular bone variables, and 0.2-1.04% for the cortical bone variables was set in the Bone Density Research Laboratory (Appendix D).

1RM Strength Testing

The leg press machine (Cybex, Medway, MA) was used for this study. Trained personnel instructed each participant on correct techniques for the two leg press. Following the instruction, participants practiced at light intensities to get accustomed to the machine. Participants were instructed to warm up for 3 minutes at a comfortable pace on a stationary bicycle ergometer (828E, Monark). Participants were then instructed to sit in a semi-reclined position on a Cybex two-leg press weight machine, where they completed a set of 5-10 with a load approximately equal to 50% of their estimated maximal strength. Following a 1 minute rest, participants then completed 3-5 sets with a load approximately equal to 75% of their estimated maximal strength. Then, following about 2 minutes rest, loads were increased so that a maximal voluntary effort was achieved within 5 more attempts. The maximum effort is to define as the maximum load that subject could lift in a single attempt with a complete range of motion. Two minutes rest were given between the lifts, and the 1RM was obtained within 5 attempts.

Jump Test Measurement

Jump power and velocity were assessed by having the participants perform a jump test on the jump mat (Just Jump, Probotic, AL) with a Tendo FiTRODYNE power

and a speed analyzer (Tendo Sports Machines, Trencin, Slovak Republic). Familiarization was done prior to the actual test, where participants practiced to get accustomed to the countermovement jump.

Participants' body weight with their shoes and transfer belt on was measured. Participants were asked to step on the mat, stand with feet shoulder-width apart, and performed 3 countermovement vertical jumps by crouching, then jumping with non-restricted arm motion, and then landing on the jump mat. The power (watts) and jump velocity (meters/second) from the Tendo machine, and the time in the air (seconds) and jump height (inches) from the Just Jump device were recorded. A minimum of 1 minute rest or as long as the subject needed was allowed between jumps. Trained spotters stood on either side of the participant to help with balance if needed. Additionally, a transfer belt was fastened around the waist of the participant that was held by the spotter to stabilize the participant if she lost her balance. A total of 3 counter movement jumps were recorded for each participant and the average of the 3 trials for all the variables were used for data analyses. The precision for jump performance and 1RM strength along with intraclass coefficient (ICC) is listed in Table 1.

Table 1. Precision for Jump Test Variables (n=15)

Variable	ICC	CV%
Jump Power (watts)	0.94	3.74%
Jump velocity (m/s)	0.80	4.40%
Air Time (sec)	0.96	2.06%
Vertical Jump Height (inches)	0.97	3.06%
1RM Strength (kg)	0.98	1.94%

Blood Sampling and Biochemical Assays

A blood sample (7.5 ml) was collected via venipuncture by a phlebotomist at the Goddard Health Center in the morning (8:00-9:00 am). Participants were instructed to fast for 8 hours before the blood collection. Blood samples were allowed to clot, centrifuged and serum samples were transferred into 6 microtubes. The plasma samples were kept frozen at -84°C prior to the assays.

Prior to each assay, the frozen serum samples and all kits reagents were taken out of the freezer and allowed to reach room temperature. Thawing of the frozen samples was done only once to avoid protein denaturation. Commercial Enzyme-Linked TECO medical Immunoassay Kit (Quidel Corp., Santa Clara, CA) was used to measure serum levels of sclerostin. Human DKK-1 ELISA (Eagle Biosciences, Inc, Nashua, NH) was used to measure serum levels of DKK-1. Serum FSH (Alpco, Salem, NH) was measured to confirm the perimenopausal status only in middle-aged women. We found FSH concentrations were less than 15 IU/L, confirming that all subjects were premenopausal women.

Step-by-step protocols were followed per assay kit instruction manuals (see Appendix G). Standard precautions were applied when handling bodily fluids. Assay precision was determined by measuring control samples at the beginning and end of each assay (intra-assay) and by measuring the same control sample in each assay run (inter-assay). For good precision, both intra- and inter-assay coefficients of variation (CV%) should be less than 10%. In this study, the intra- and inter-assay CVs for sclerostin were 3.7 and 8.3%; the intra- and inter-assay CVs for DKK-1 were 4.9 and 5.1%, respectively.

Data Analyses

Data were analyzed using IBM SPSS 23.0 (SPSS Inc., Chicago, IL). All descriptive statistics are reported as means ± standard deviation (SD) unless otherwise stated. All dependent variables were tested for normality using the Kolmogorov-Smirnov test. Two-way ANOVA (age × physical activity status) was used to determine the effects of age and physical activity status on sclerostin and DKK-1. If confounding variables such as body weight, height, and BMC were significantly different between the age groups (as determined by independent t-tests), then these variables were used as covariates in ANCOVAs. Zero-order Pearson Product Moment correlation coefficients were computed to determine the relationships between sclerostin/DKK-1 and aBMD, vBMD, leg strength, and jump power, respectively, for all 50 participants combined. Intraclass correlation coefficients (ICC) were calculated for jump test and leg press variables. The level of significance was set at $p \leq 0.05$.

Chapter 4: Results and Discussion

The purpose of this study was to compare serum concentrations of sclerostin and DKK-1 in young (20-30 years old) and middle-aged (35-45 years old) premenopausal women. These age groups were selected to allow comparisons between women who are still accruing bone mass versus those who have already achieved their peak bone mass. The present study also evaluated the differences in sclerostin and DKK-1 concentrations based on the physical activity status (low, moderate, and HEPA-active). Dependent variables included the Wnt signaling inhibitors, sclerostin and DKK-1, and BMD and BMC of total body, lumbar spine, and dual femur. This study also measured vBMC and vBMD of non-dominant tibia (4%, 38%, and 66%) sites. Additional variables included components of hip structural analysis (HSA), and lower body strength (leg press) and jump power. The secondary purposes of this study were to evaluate sclerostin and DKK-1 relationships with aBMD, vBMD, HSA variables, and muscle performance variables.

Participants

In this cross-sectional study, participants were premenopausal women in the age groups of 20-30 and 35-45 years. A total of 57 women responded to study advertisements and were screened for eligibility. Five women were excluded prior to enrollment due to irregular menstruation or medications, and 2 women were lost to follow-up. A total of 50 young (n=25) and middle-aged (n=25) women participated in this study and completed all protocols. Participants were recruited from the University of Oklahoma, Norman, and surrounding areas. Recruitment methods for this study include flyers, mass email, and word-of-mouth. Participants also reported the details on

past history of taking hormonal contraception. Based on this information, participants were further categorized into two groups: hormonal contraceptive users (HC users) (n=14) and non-users (n=36). Of the 14 contraceptive-using participants, 8 participants were using oral contraceptives with dosages of estradiol greater than 20 μg, 5 were using an intrauterine device, and 1 reported using depo injection.

Table 2 summarizes the physical characteristics of participants for the two age groups. There was a trend for young women being taller than middle-aged women ($p=0.052$). The total physical activity scores were not significantly different between age groups ($p>0.46$). Since there were no statistically significant differences for confounding variables such as height, weight, percent body fat, fat mass, fat-free mass and bone-free lean body mass, no covariates were used in the primary data analyses.

Table 2. Physical Characteristics for Young and Middle-aged Groups (Mean ± SD)

Variable	Young (n=25)	Middle-Aged (n=25)
Age (yrs)*	25.56 ± 3.35	39.00 ± 2.49
Height (cm)	167.56 ± 5.45	164.43 ± 5.66
Weight (kg)	67.16 ± 13.23	66.21 ± 12.30
Calcium Intake (mg/day)	902 ± 411	875 ± 387
pBPAQ Score	55.58 ± 48.67	55.42 ± 56.24
cBPAQ Score	7.11 ± 8.57	14.56 ± 26.20
Total PA Score (mets/min)	3241.98 ± 2671.95	3783.64 ± 2578.51
Body Fat %	31.47 ± 6.66	34.08 ± 9.05
Fat Mass (kg)	20.85 ± 8.09	24.85 ± 12.23
BFLBM (kg)	43.95 ± 7.31	41.46 ± 5.91
Fat Free Mass (kg)	46.42 ± 7.55	43.82 ± 6.14

* $p<0.05$ significant age difference; pBPAQ= past Bone Specific Physical Activity Questionnaire; cBPAQ= current Bone Specific Physical Activity Questionnaire; Total PA Score= Total Physical Activity Score; BFLBM= Bone Free Lean Body Mass.

Physical Activity, Serum Sclerostin, and Serum DKK-1

The current study utilized IPAQ to assess physical activity scores. The three levels of physical activity were low (less than 600 MET-min/week), moderate (MET-min/week between 600 and 2,999 MET-min/week), HEPA-active (more than 3000 MET-min/week). In the current study, only three participants self-reported as a low-level physical activity, therefore, we combined low and moderate activity together. Two-way ANOVA was used to determine whether there was an interaction between age

group and physical activity status on sclerostin and DKK-1 levels. Serum sclerostin concentrations were significantly higher in middle-aged women compared to young women ($p<0.001$) (Table 3, Figure 4). The mean values of sclerostin for young and middle-aged were 0.41 ± 0.01 ng/ml and 0.54 ± 0.01 ng/ml, respectively (Figure 4), which is within the range expected for premenopausal women according to the assay kit (Appendix F). There was also a significant main effect for physical activity levels ($p<0.05$), with serum sclerostin concentrations being significantly higher in HEPA–active women compared to low-moderate active women (Table 3). However, there was no age × physical activity level interaction for sclerostin concentrations ($p>0.05$). Since total body aBMD was higher in HEPA-active women compared to low-moderately active women, total body aBMD was used as a covariate, which eliminated the main effect for physical activity levels ($p= 0.11$).

Serum DKK-1 concentrations were not significantly different between age groups or based on physical activity classifications ($p>0.05$). The mean values of serum DKK-1 for young and middle-aged women were 54.25 ± 3.2 pmol/l and 48.77 ± 3.2 pmol/l, respectively (Table 3, Figure 5). Serum DKK-1 concentration ranged from 15.53 to 86.16 pmol/l. Due to lack of literature on the normal ranges, we are not able to compare our DKK-1 ranges with other studies.

Independent t-tests were used to assess differences between HC users and non-users in sclerostin and DKK-1 levels. There was a significant difference between HC users and non-users for both sclerostin and DKK-1 concentrations ($p<0.05$). The mean values of sclerostin for HC users ($n=14$) and non-users ($n=36$) were 0.42 ± 0.02 ng/ml and 0.50 ± 0.01 ng/ml, respectively. The mean values for DKK-1 levels for HC users

(n=14) and non-users (n=36) were 42.38 ± 3.9 and 55.06 ± 2.6 pmol/l, respectively. We also compared differences between oral contraceptive users (OC users) (n=8) and non-users (n=42) and found that there were significant differences between the two groups for sclerostin concentrations (p=0.05) and no significant differences for DKK-1 concentrations but the trend was for young women to have higher levels (p=0.07).

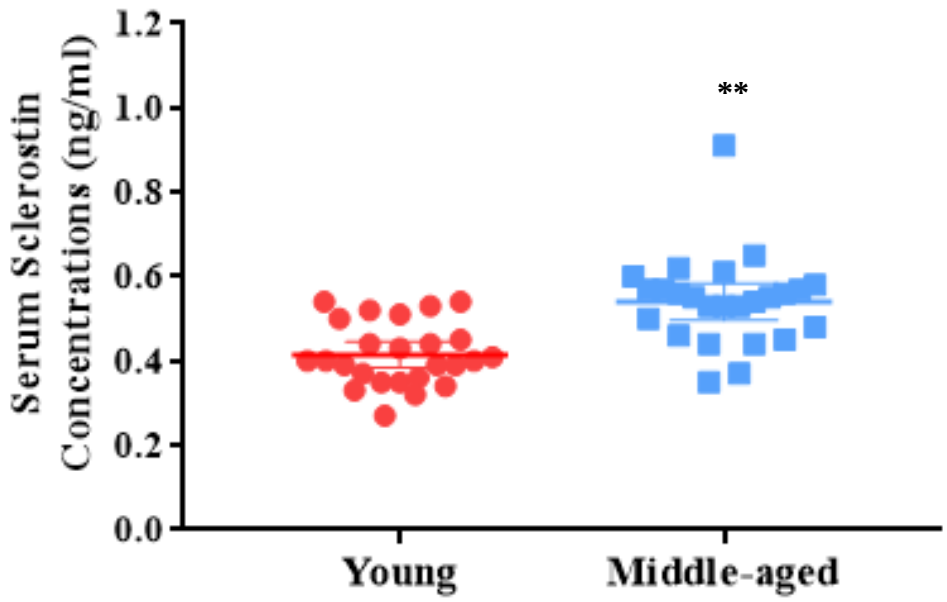


Figure 4. Serum Sclerostin Concentrations in Young (n=25) and Middle-aged (n=25) Women (Mean ± 95% CI). ** Significant age effect p<0.01

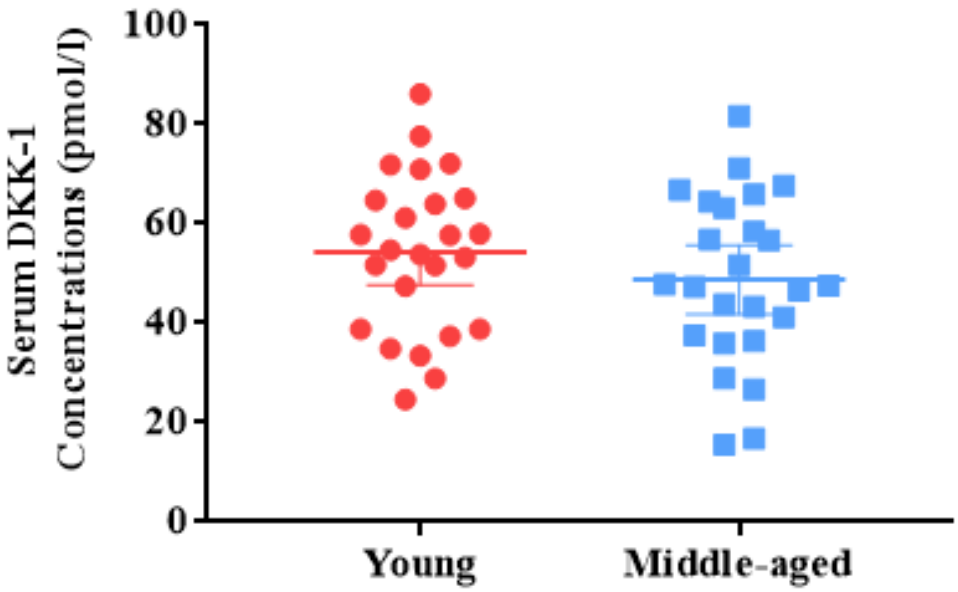


Figure 5. Serum DKK-1 Concentrations in Young (n=25) and Middle-aged (n=25) women (Mean ± 95% CI).

Table 3. Serum Sclerostin and DKK-1 Based on Age and Physical Activity Levels (Mean ± SD)

Variables	Young (n=25)		Middle-Aged (n=25)	
	Low-Moderate (n=13)	HEPA-Active (n=12)	Low-Moderate (n=12)	HEPA-Active (n=13)
Sclerostin (ng/ml)**†	0.38 ± 0.77	0.44 ± 0.06	0.51 ± 0.09	0.56 ± 0.01
DKK-1 (pmol/l)	55.36 ± 17.14	53.05 ± 14.99	48.70 ± 17.95	48.84 ± 16.52

** Significant age effect $p<0.01$; † Significant physical activity effect $p<0.05$

Areal Bone Mineral Density

Two-way ANOVA was used to determine if there was an interaction between age group and physical activity levels on aBMD variables. There were significant main effects for age ($p<0.05$) and physical activity levels, however, there was no age × physical activity levels interaction observed for aBMD variables. Both age groups had average Z-Scores above -2.0 for total body and spine. However, one participant had a Z-Score below -2.0 for right trochanter aBMD.

There was a significant main effect of physical activity levels for total body aBMD, total body Z-scores, and spine aBMD ($p<0.05$), with HEPA-active women having higher mean values for total aBMD, total body Z-Score, and spine L1-L4 aBMD (Table 4). There was also a significant main effect of age for spine BMC, with the young group having significantly higher BMC than middle-aged women ($p<0.05$). However, there were no significant age × physical activity level interactions for total body and lumbar spine aBMD variables. There were significant main effects of physical activity levels for of all the hip aBMD variables ($p<0.05$). HEPA-active women had significant higher values for hip aBMD variables compared to low-moderate active women ($p<0.05$) (Table 5). There was also a significant main effect of age for left trochanter aBMD, with the young group having significantly higher left trochanter

aBMD than middle-aged women ($p<0.05$). There were no significant age × physical activity level interactions for hip aBMD variables.

Table 4. Total Body and Lumbar Spine Areal Bone Mineral Density (Mean ± SD)

Variables	Young (n=25)		Middle-Aged (n=25)	
	Low-Moderate (n=13)	HEPA-Active (n=12)	Low-Moderate (n=12)	HEPA-Active (n=13)
Total Body aBMD (g/cm^2)†	1.192 ± 0.555	1.262 ± 0.086	1.188 ± 0.108	1.221 ± 0.082
Total Body BMC (g)	2427.38 ± 195.30	2554.41 ± 337.80	2337.16 ± 320.43	2377.61 ± 331.79
Total Body Z-Scores †	1.01 ± 0.60	1.51 ± 0.54	0.94 ± 1.13	1.40 ± 0.72
Spine L1-L4 aBMD (g/cm^2)†	1.228 ± 0.078	1.346 ± 0.129	1.235 ± 0.122	1.255 ± 0.126
Spine L1-L4 BMC*	68.04 ± 6.45	73.51 ±10.50	64.83 ± 10.22	65.65 ± 10.41
Spine L1-L4 Z-Scores	0.34 ± 0.76	1.25 ± 1.21	0.37 ± 1.10	0.60 ± 0.97

*Significant age effect $p<0.05$; † Significant physical activity effect $p<0.05$; aBMD= areal Bone Mineral Density.

.

Table 5. Hip Areal Bone Mineral Density (Mean ± SD)

Variables	Young (n=25)		Middle-Aged (n=25)	
	Low-Moderate (n=13)	HEPA-Active (n=12)	Low-Moderate (n=12)	HEPA-Active (n=13)
Right FN aBMD (g/cm^2) †	1.009 ± 0.087	1.122 ± 0.085	0.984 ± 0.136	1.053 ± 0.100
Left FN aBMD (g/cm^2) †	1.018 ± 0.088	1.116 ± 0.083	0.993 ± 0.161	1.054 ± 0.099
Right FN Z-Scores†	-0.27 ± 0.58	0.50 ± 0.70	-0.06 ± 0.99	0.51 ± 0.73
Left FN Z-Scores†	-0.20 ± 0.50	0.41 ± 0.66	0.00 ± 1.15	0.52 ± 0.75
Right Troch aBMD (g/cm^2) †	0.811 ± 0.069	0.898 ± 0.076	0.778 ± 0.119	0.856 ± 0.089
Left Troch aBMD (g/cm^2) *†	0.816 ± 0.067	0.887 ± 0.082	0.758 ± 0.126	0.833 ± 0.086
Right Troch Z-Scores †	-0.40 ± 0.60	0.33 ± 0.76	-0.49 ± 1.05	0.25 ± 0.81
Left Troch Z-Scores †	-0.38 ± 0.52	0.26 ± 0.74	-0.65 ± 1.09	0.06 ± 0.83
Left THIP aBMD (g/cm^2) †	1.025 ± 0.089	1.105 ± 0.074	0.984 ± 0.150	1.054 ± 0.080
Right THIP aBMD (g/cm^2) †	0.811 ± 0.069	0.898 ± 0.076	0.778 ± 0.119	0.856 ± 0.089
Right THIP Z-Scores †	-0.04 ± 0.64	0.77 ± 0.66	0.07 ± 1.02	0.73 ± 0.71
Left THIP Z-Scores †	0.05 ± 0.60	0.64 ± 0.66	-0.04 ± 1.12	0.59 ± 0.68

* Significant age effect $p<0.05$; † Significant physical activity effect $p<0.05$; BMD= Bone Mineral Density; Troch= Trochanter; FN= Femoral Neck; THIP= Total Hip

Volumetric Bone Mineral Density

The variables measured by pQCT were total volumetric BMC, total volumetric BMD, trabecular BMD, trabecular volumetric BMD, total area, trabecular area, periosteal circumference, endosteal circumference, stress-strain index, cortical volumetric BMC, cortical volumetric BMD, and polar moment of inertia at 4%, 38% and 66% of the non-dominant tibia. Muscle cross-sectional area was also measured at 66% of the non-dominant tibia.

Two-way ANOVA was used to determine the effects of age group and physical activity status on pQCT bone density variables. There were significant main effects for age;

however, there were no significant main effects for physical activity status or age × physical activity level interactions for pQCT bone density variables. Table 6 shows significant main effects for age for total volumetric BMC, trabecular BMC and BMD, and trabecular bone strength index at the 4% tibia site ($p<0.05$), which were higher in young women compared to middle-aged women.

Table 7 shows that there was a significant main effect of age for cortical vBMD at the 38% tibia site ($p<0.05$). Middle-aged women had significantly higher cortical vBMD than young women ($p<0.05$). There were no significant main effects for physical activity levels and also no significant age × physical activity interaction for 38% pQCT bone characteristics. Table 8 shows that there was a significant main effect of age for total area, Peri C, SSI, and IPolar at the 66% tibia site ($p<0.05$). Young women had significantly greater total area, Peri C, SSI and IPolar compared to middle-aged women ($p<0.05$). There were no significant main effects for physical activity level and also no significant age × physical activity interactions for 66% pQCT bone characteristics.

Table 6. pQCT 4% Bone Characteristics of the Non-dominant Tibia (Mean ± SD)

Variables	Young (n=25)		Middle-Aged (n=25)	
	Low-Moderate (n=13)	HEPA-Active (n=12)	Low-Moderate (n=12)	HEPA-Active (n=13)
Total vBMC (mg/mm) *	294.07 ± 39.20	323.04 ± 46.26	277.44 ± 50.05	281.83 ± 41.06
Total vBMD (mg/cm^3)	304.046 ± 34.380	317.475 ± 30.536	287.041 ± 27.047	310.023 ± 37.503
Trab BMC (mg/mm) *	212.73 ± 36.81	233.23 ± 39.02	194.94 ± 38.24	194.45 ± 34.51
Trab vBMD (mg/cm^3) *	253.907 ± 35.037	269.475 ± 32.293	233.233 ± 23.522	249.876 ± 38.410
Total Area (mm^2)	969.04 ± 89.84	1020.92 ± 137.47	962.18 ± 105.94	910.32 ± 97.47
Trab Area (mm^2)	838.21 ± 85.76	880.79 ± 126.75	830.61 ± 93.31	778.60 ± 88.99
Peri C (mm)	109.63 ± 6.05	113.02 ± 7.78	109.80 ± 6.01	106.81 ± 5.78
BSI (mg*mm)	90.30 ± 20.97	103.08 ± 20.90	80.68 ± 21.81	88.42 ± 21.96
Trab BSI (mg*mm)**	55.61 ± 16.62	64.34 ± 14.97	46.20 ± 13.87	49.58 ± 14.93

* Significant age effect $p<0.05$; ** Significant age effect $p<0.001$; vBMD= volumetric Bone Mineral Density; Trab= Trabecular; vBMC= volumetric Bone Mineral Content; Peri C= Periosteal Circumference; BSI= Bone Strength Index

Table 7. pQCT 38% Bone Characteristics of the Non-dominant Tibia (Mean ± SD)

Variables	Young (n=25)		Middle-Aged (n=25)	
	Low-Moderate (n=13)	HEPA-Active (n=12)	Low-Moderate (n=12)	HEPA-Active (n=13)
Total vBMC (mg/mm)	350.65 ± 52.98	358.94 ± 55.80	324.33 ± 41.26	342.04 ± 47.04
Total vBMD (mg/cm^3)	950.223 ± 76.200	960.041 ± 51.449	947.358 ± 91.100	975.492 ± 36.466
Total Area (mm^2)	368.59 ± 45.51	373.60 ± 50.91	353.46 ± 50.86	350.85 ± 47.95
Peri C (mm)	67.93 ± 4.25	68.36 ± 4.73	65.55 ± 2.87	66.25 ± 4.64
Endo C (mm)	33.00 ± 4.78	32.11 ± 3.70	32.03 ± 5.28	31.05 ± 2.86
Cort vBMC (mg/mm)	334.70 ± 51.51	346.91 ± 54.52	312.44 ± 41.89	330.58 ± 45.93
Cort vBMD*	1194.684 ± 19.640	1195.108 ± 15.482	1206.775 ± 26.952	1209.453± 13.983
Cort Area	280.209 ± 42.74	290.50 ± 46.83	258.90 ± 34.28	273.48 ± 38.97
SSI (mm^3)	1560.23 ± 290.96	1617.98 ± 313.06	1418.00 ± 182.01	1481.689 ± 279.10
IPolar (mm^4)	23298.9 ± 562.7	24505.5 ± 7214.8	20173.5 ± 3661.1	21604.9 ± 5559.3

* Significant age effect $p<0.05$; vBMD= volumetric Bone Mineral Density; vBMC= volumetric Bone Mineral Content; Cort= cortical; Peri C= Periosteal Circumference; Endo C= Endosteal Circumference; SSI= Stress Strain Index; IPolar= Polar Moment of Inertia of the Cortical Bone

Table 8. pQCT 66% Bone Characteristics of the Non-dominant Tibia (Mean ± SD)

Variables	Young (n=25)		Middle-Aged (n=25)	
	Low-Moderate (n=13)	HEPA-Active (n=12)	Low-Moderate (n=12)	HEPA-Active (n=13)
Total vBMC (mg/mm)	378.12 ± 47.69	377.29 ± 63.71	350.66 ± 44.13	353.22 ± 59.49
Total vBMD (mg/cm^3)	698.700 ± 54.307	696.842 ± 58.352	708.833 ± 78.109	732.077 ± 102.475
Total Area (mm^2)*	542.80 ± 70.86	543.13 ± 92.38	497.44 ± 49.66	501.03 ± 63.83
Peri C (mm)*	82.42 ± 5.40	82.33 ± 7.09	78.89 ± 3.98	79.17 ± 5.38
Endo C (mm)	55.55 ± 5.41	55.13 ± 6.60	52.57 ± 5.13	52.60 ± 5.06
Cort vBMC (mg/mm)	341.16 ± 42.63	341.61 ± 55.61	319.87 ± 41.79	323.78 ± 54.11
Cort vBMD	1156.546 ± 22.274	1148.467 ± 22.026	1164.817 ± 28.665	1161.608 ± 17.160
Cort Area	295.07 ± 36.89	298.01 ± 51.20	274.60 ± 35.31	278.94 ± 47.38
SSI (mm^3)*	2347.68 ± 440.48	2434.80 ± 574.20	2088.79 ± 290.81	2140.60 ± 411.16
IPolar (mm^4)*	42757.9 ± 9987.9	43968.3 ±13883.3	36204.1 ± 6845.7	37694.6 ± 9923.7
MSCA	6364.42 ± 941.00	7253.52 ± 1506.85	6616.92 ± 1231.5	6843.48 ± 1365.79

*Significant age effect $p<0.05$; vBMD= volumetric Bone Mineral Density; vBMC= volumetric Bone Mineral Content; Cort= cortical; Peri C= Periosteal Circumference; Endo C= Endosteal Circumference; SSI= Stress Strain Index; IPolar= Polar Moment of Inertia of the Cortical Bone; MSCA= Muscle Cross-Sectional Area

Hip Structural Analyses

The measurement variables for hip structural analyses included right and left SI, BR, SM, and CSMI.

Two-way ANOVA was used to determine the effects of age group and physical activity status on hip structural analyses variables. Table 9 shows that a significant main effect of age was observed for left SM ($p<0.05$). Middle-aged women had significantly lower SM compared to young women ($p<0.05$). There were also significant main effects of age ($p<0.05$) and physical activity level ($p<0.01$) for right BR. Middle-aged women

had significantly higher BR compared to young women ($p<0.05$) and HEPA-active women had significantly lower BR compared to low-moderately active women ($p<0.01$). However, there were no age × physical activity level interactions for hip structural analyses variables.

Table 9. Hip Structural Analyses (Mean ± SD)

Variables	Young (n=25)		Middle-Aged (n=25)	
	Low-Moderate (n=13)	HEPA-Active (n=12)	Low-Moderate (n=12)	HEPA-Active (n=13)
Right SI	1.59 ± 0.53	1.73 ± 0.39	1.40 ± 0.28	1.63 ± 0.36
Left SI	1.63 ± 0.34	1.72 ± 0.43	1.50 ± 0.38	1.56 ± 0.37
Right SM (mm^3)	659.12 ± 110.87	702.56 ± 138.86	614.98 ± 110.29	614.53 ± 116.30
Left SM (mm^3)*	651.60 ± 84.35	714.91 ± 139.36	627.12 ± 129.90	593.78 ± 93.85
Right BR*††	3.04 ± 0.68	2.05 ± 0.52	3.40 ± 1.22	2.98 ± 0.99
Left BR	2.90 ± 0.98	2.86 ± 1.07	3.11 ± 1.14	3.40 ± 1.00
Right CSMI (mm^4)	10310.00 ± 2798.03	10671 ± 2924.34	9397.16 ± 2188.16	9393.84 ± 2151.18
Left CSMI (mm^4)	9914.53 ± 1898.80	11041.83 ± 2857.46	9690.83 ± 2457.32	8986.53 ± 1565.10

* Significant age effect $p<0.05$; †† Significant physical activity levels $p<0.001$; SI= Strength Index; SM= Sectional Modulus; BR= Buckling Ratio; CSMI= Cross-sectional Moment of Inertia.

Muscle Function Assessment

The measurement variables for muscle function were jump performance and 1RM leg strength. The jump performance variables were time in air, velocity, vertical jump height, jump power and relative jump power.

Two-way ANOVA was used to determine the effects of age group and physical activity status on muscle function assessment. A significant main effect of age was observed for time in air, jump height, relative jump power, and 1RM ($p<0.05$), with middle-aged women having significantly lower values compared to young women

($p<0.05$). There was no main effect of physical activity level and no age × physical activity level interactions for those variables.

Table 10. Muscle Performance Variables (Mean ± SD)

Variables	Young (n=25)		Middle-Aged (n=25)	
	Low-Moderate (n=13)	HEPA-Active (n=12)	Low-Moderate (n=12)	HEPA-Active (n=13)
Time in air (s)**	0.51 ± 0.06	0.53 ± 0.05	0.47 ± 0.05	0.47 ± 0.06
Jump height (inches)**	13.43 ± 3.10	14.13 ± 2.69	11.12 ± 2.52	11.20 ± 3.33
Velocity (m/s)	0.99 ± 0.13	1.04 ± 0.09	0.94 ± 0.13	0.92 ± 0.09
Jump Power (watts)	647.40 ± 107.99	698.13 ± 141.61	622.91 ± 142.39	593.20 ± 127.62
Rel Jump Power (watts/kg)*	9.85 ± 1.30	11.44 ± 3.69	9.22 ± 1.49	9.17 ± 1.02
1RM (kg)*	125.17 ± 25.07	144.29 ± 36.90	113.24 ± 20.18	121.67 ± 29.23

* Significant age effect $p<0.05$; ** Significant age effect $p<0.001$; Rel =Relative

Correlations

Zero-order Pearson Product Moment correlation coefficients were computed to determine the relationships of sclerostin and DKK-1 levels with aBMD, vBMD, hip structural analyses, and muscle strength variables. Generally, all the correlation values were significantly low except for the cortical vBMD at the 38% tibia site ($r=0.5$). There was also a trend of a low positive relationship between serum concentrations of sclerostin and DKK-1 ($r= 0.22$; $p=0.058$).

There were significant but low positive relationships between total body Z-score, spine BMD, and spine Z-Scores with serum sclerostin concentrations ($p<0.05$) (Table 11). Similarly, significant but low positive relationships were found for spine BMD and spine Z-Scores with serum DKK-1 concentrations ($p<0.05$).

There were significant but low positive relationships observed between right neck Z-score, left neck Z-score, right trochanter BMD, right trochanter Z-score, left

trochanter Z-score, total right BMD, total left Z-score, and total right Z-Score with serum sclerostin concentrations ($p<0.05$) (Table 12). Similarly, significant low positive relationships were found for right trochanter BMD, left trochanter BMD, right trochanter Z-score, and left trochanter Z-score with serum DKK-1 concentrations ($p<0.05$). There were no significant correlations for sclerostin and DKK-1 with hip structural analyses variables ($p>0.05$). Similarly, no significant correlations were found for 4% pQCT bone variables with sclerostin and DKK-1 ($p>0.05$) (Appendix E).

There were significant but low negative relationships observed for total area, periosteal circumference, and endosteal circumference at 38% tibia site with serum sclerostin concentrations ($p<0.05$) (Table 13). There was also a moderate positive relationship between cortical vBMD and serum sclerostin concentrations ($p<0.001$). There were no significant relationships between 38% pQCT variables and serum DKK-1 concentrations ($p>0.05$).

There was a significant but low positive relationship between cortical vBMD at the 66% tibia site and serum sclerostin concentrations ($p<0.01$) (Table 14). There was no significant association between 66% tibia variables and serum DKK-1 concentrations.

There was a significant but low negative relationships between time in air and vertical jump height with serum sclerostin concentrations ($p<0.05$) (Table 15). No significant correlations were observed for muscle strength variables and serum DKK-1 concentrations.

Table 11. Correlations between Serum Sclerostin and DKK-1 Concentrations with Total Body and Lumbar Spine Areal BMD Variables

Variables	r	Variables	r
Serum sclerostin		Serum DKK-1	
Total Body BMD	0.18	Total Body BMD	0.11
Total Body BMC	0.05	Total Body BMC	0.14
Z-Score	0.28*	Z-Score	0.01
Spine L1-L4 BMD	0.31*	Spine L1-L4 BMD	0.28*
Spine L1-L4 BMC	0.16	Spine L1-L4 BMC	0.22
Spine L1-L4 Z-Score	0.36*	Spine L1-L4 Z-Score	0.25*

*$p<0.05$; BMD= Bone Mineral Density; BMC= Bone Mineral Content;

Table 12. Correlations between Serum Sclerostin and DKK-1 Concentrations with Hip Areal BMD Variables.

Variables	r	Variables	r
Serum sclerostin and		Serum DKK-1 and	
Right Neck BMD	0.22	Right Neck BMD	0.11
Left Neck BMD	0.21	Left Neck BMD	0.09
Right Neck Z-Score	0.46*	Right Neck Z-Score	0.09
Left Neck Z-Score	0.44*	Left Neck Z-Score	0.06
Right Trochanter BMD	0.25*	Right Trochanter BMD	0.27*
Left Trochanter BMD	0.16	Left Trochanter BMD	0.26*
Right Trochanter Z-Score	0.36*	Right Trochanter Z-Score	0.27*
Left Trochanter Z-Score	0.30*	Left Trochanter Z-Score	0.26*
Total Left BMD	0.15	Total Left BMD	0.18
Total Right BMD	0.25*	Total Right BMD	0.14
Total Left Z-Score	0.32*	Total Left Z-Score	0.16
Total Right Z-Score	0.42**	Total Right Z-Score	0.12

*p<0.05; **p<0.01; BMD= Bone Mineral Density

Table 13. Correlations between Serum Sclerostin and DKK-1 Concentrations with 38% pQCT Variables.

Variables	r	Variables	r
Serum sclerostin and		Serum DKK-1 and	
Total BMC	-0.09	Total BMC	0.11
Total vBMD	0.26*	Total vBMD	0.10
Total Area	-0.24*	Total Area	0.11
Periosteal Circumference	-0.24*	Periosteal Circumference	0.06
Endosteal Circumference	-0.31*	Endosteal Circumference	-0.03
Cortical BMC	-0.05	Cortical BMC	0.11
Cortical vBMD	0.50**	Cortical vBMD	0.19
Cortical Area	-0.10	Cortical Area	0.09
Stress-Strain Index	-0.15	Stress-Strain Index	0.14
IPolar	-0.18	IPolar	0.11

*p<0.05; **p<0.01; vBMD= Volumetric Bone Mineral Density; vBMC= volumetric Bone Mineral Content; IPolar= Polar Moment of Inertia

Table 14. Correlations between Serum Sclerostin and DKK-1 Concentrations with 66% pQCT Variables.

Variables	r	Variables	r
Serum sclerostin and		Serum DKK-1 and	
Total BMC	-0.13	Total BMC	0.13
Total vBMD	0.12	Total vBMD	0.04
Total Area	-0.20	Total Area	0.09
Periosteal Circumference	-0.20	Periosteal Circumference	0.08
Endosteal Circumference	-0.18	Endosteal Circumference	0.02
Cortical BMC	-0.09	Cortical BMC	0.14
Cortical vBMD	0.34**	Cortical vBMD	0.17
Cortical Area	-0.13	Cortical Area	0.11
Stress-Strain Index	-0.15	Stress-Strain Index	0.11
IPolar	-0.16	IPolar	0.14
Muscle Cross Sectional Area	0.31	Muscle Cross Sectional Area	0.02

**p<0.01; vBMD= Volumetric Bone Mineral Density; BMC= Bone Mineral Content; IPolar= Polar Moment of Inertia

Table 15. Correlations between Serum Sclerostin and DKK-1 Concentrations with Muscle Performance Variables

Variables	r	Variables	r
Serum sclerostin and		Serum DKK-1 and	
Time in air	-0.27*	Time in air	-0.15
Vertical Jump height	-0.27*	Vertical Jump height	-0.02
Velocity	-0.20	Velocity	-0.04
Power	-0.18	Power	0.03
1RM	-0.06	1RM	0.16
Relative Power	-0.18	Relative Power	-0.01

*$p<0.05$

Discussion

The current study was designed to investigate the serum concentrations of sclerostin and DKK-1 in young and middle-aged premenopausal women. The selection of these age ranges allowed us to compare these two Wnt inhibitors between women who were still accruing bone mass versus those who had already achieved their peak bone mass. The present study also evaluated the differences in the sclerostin and DKK-1 concentrations based on physical activity status: low-moderate versus HEPA-active. Furthermore, this study evaluated the relationships between serum sclerostin and DKK-1 with several BMD and muscle performance variables. The unique characteristic of this study was the investigation of Wnt signaling inhibitor levels with respect to peak bone mass. The results showed significant differences in sclerostin levels based on peak bone mass and physical activity level. Sclerostin and DKK-1 levels and their relationships to mechanical loading have been well documented in previous animal model studies. However, human research is limited and there have been conflicting results. The findings from the current investigation provide insights into the relationships between Wnt signaling inhibitor characteristics, peak bone mass, and physical activity in premenopausal women.

It is well documented that mechanical loading or physical activity influence bone remodeling processes through the activation of osteoblasts and osteoclasts; more recent findings also implicate a regulatory role of osteocytes in this process. Osteocytes are the principle cells in integrating mechanical and chemical signals. Sclerostin and DKK-1 are exclusively found in osteocytes and act as negative regulators of bone metabolism. Therefore, understanding mechanical loading or physical activity and

expression of sclerostin and DKK-1 provides important cues for skeletal mechanoregulation.

Age, Physical Activity, and Wnt Signaling Inhibitors

The Wnt signaling pathway is fine-tuned by several secreted glycoproteins such as sclerostin and DKK-1, which bind to the LRP5/6 receptor and disrupt the canonical Wnt signaling pathway. This signaling pathway is an important regulator of skeletal adaptation based on individual biomechanical demands. In animal models, mechanical stimulation of bone effectively decreases osteocyte production of sclerostin and DKK-1 in a dose-dependent manner, while their levels are increased during unloading or bed rest condition (Robling et al., 2006; Gaudio et al., 2012).

The major finding from the current study is that serum sclerostin levels were significantly higher in middle-aged women compared to young women, which is in line with the previous study by Modder et al. (2010), where serum sclerostin concentrations significantly increased with age. The previous study had larger sample size compared to the current study and men had significantly higher levels of sclerostin than women. The authors explained that the higher serum sclerostin in men could possibly be due to higher skeletal mass, as total body BMC was significantly higher in men. In the present study, men were excluded and no significant differences were observed for total body BMC and BFLBM between the two age groups of women.

Another study reported that sclerostin levels increased linearly with age, and that postmenopausal women had significantly higher sclerostin levels than premenopausal women (Ardawi et al., 2011). Age-related impairment in bone formation and decreased renal function could be possible mechanisms accounting for the increased circulating

levels of sclerostin with aging. Furthermore, aging is associated with an increase in oxidative stress via the FoxO mediated pathway, which could possibly lead to attenuation of Wnt proteins and osteoblasts, thus increasing serum sclerostin levels (Manolaga, 2007; Lopez-Otin, 2013).

Only a few studies in humans have explored circulating DKK-1 levels (Gaudio et al., 2010; Tian et al., 2015), thus necessitating exploring animal studies to understand Wnt signaling inhibitors in human skeletal metabolism. The evidence from animal studies using overexpressed DKK-1 and DKK-1 haploinsufficient mice suggest that DKK-1 is a potent inhibitor of bone formation (Li et al., 2006; Morvan et al., 2006). Furthermore, animal studies suggest that DKK-1 levels were very low in healthy tissue compared to necrotic tissue (Kinsley et al., 2015).

The current study found no significant differences in serum DKK-1 between the two age groups. In contrast, Dovjak et al. (2014) compared DKK-1 in young and older men and women and found that DKK-1 levels were higher in older men and women compared to younger individuals, suggesting the importance of Wnt inhibitors with aging. Tian et al. (2015) showed that serum DKK-1 levels were significantly higher in osteoporotic postmenopausal women compared to healthy postmenopausal women, suggesting a potential role of DKK-1 in skeletal metabolism and the Wnt signaling pathway. Participants in the current study were younger and were from diverse ethnicity compared to those investigated by Tian et al. (2015), possibly explaining disagreement between the two studies.

Evidence suggests that the effects of mechanical loading on bone mass is mediated by the effects of resident bone cells (osteoblasts, osteoclasts, and osteocytes). The abundance and strategic location of osteocytes make them favorable for detecting mechanical signals that lead to bone adaptation (Delgado-Calle et al., 2017). It has been reported that expression of sclerostin is higher in mature osteocytes and its expression is decreased in the presence of mechanical loading (Robling et al., 2006).

An immunohistochemical study on C57BL/6 mice showed that dynamic axial loading is associated with downregulation of sclerostin expression in osteocytes and increased bone volume and bone formation (Moustafa et al., 2011). The results from the current study showed that sclerostin levels were significantly higher in HEPA-active women compared to low-moderately active women, although we did not find significant interactions between age and physical activity levels.

In contrast to the current study, Amrein et al. (2012) found that physically active adults had significantly lower sclerostin levels. This study utilized the Baecke Questionnaire, while the present study utilized the International Physical Activity Questionnaire for assessing physical activity. Although both questionnaires were validated, physical activity scores obtained from both questionnaires have a poor positive correlation (Sadeghisani et al., 2016). Differences in the quantification of physical activity scores could explain the disagreement between the two studies. Similarly, another cross-sectional study (Gaudio et al., 2010) reported that sclerostin levels were significantly higher in immobilized elderly women compared to a control group, supporting the link between sclerostin and mechanical unloading.

In humans, the effect of exercise on sclerostin is inconsistent among intervention studies, with some studies showing an increase (Zagrodna et al., 2016), decrease (Ardawi et al., 2012; Hinton et al., 2017) or no change (Mosti et al., 2014) in sclerostin levels. The present study is not a longitudinal study and utilized a self-reported physical activity questionnaire. The HEPA-active women had significantly higher sclerostin levels compared to low-moderately active women. The following possible mechanisms could explain the sclerostin levels in HEPA-active women in the present study: 1. total body BMD was significantly higher in HEPA active women, and larger total body skeletal mass could result in releasing more sclerostin into the general circulation; and 2. an increase in Wnt signaling could stimulate an increase in sclerostin levels that provides a negative feedback signal at the basic multicellular unit so that bone homeostasis can be maintained (Baron et al., 2012).

To date, only a few studies have examined the effects of exercise on serum DKK-1 levels. Animal model studies have reported that DKK-1 expression increased with sedentary behavior and decreased with long-term exercise (Bayod et al., 2014). A cross-sectional study of immobilized elderly women and a control group reported no significant difference in serum DKK-1 levels, which is in line with the current study.

A recent intervention study reported a significant decrease in serum DKK-1 levels after 12 weeks of strength training exercise (Kim et al., 2017). The pharmacokinetics of DKK-1 in bone tissue could be different for various types of exercises and we measured serum sample not the bone tissue. Therefore, based on these results, DKK-1 responses on mechanical loading and bone homeostasis warrants further investigation.

It should be noted that in the present study we did not control the menstrual cycle phase while collecting blood samples. Previously, it has been reported that sclerostin and DKK-1 concentrations were not affected by the menstrual cycle and were not related with estradiol in premenopausal women (35-45 years) (Cidem et al., 2012; Liakou et al., 2016). Surprisingly, we found HC users had significantly lower sclerostin and DKK-1 levels compared to non-users. Therefore, the influence of sex hormones on Wnt signaling inhibitors deserves further investigation.

There are various factors that could have influenced the results of the current study, such as types of assay kits (detecting polyclonal versus monoclonal antibodies of sclerostin/DKK-1), types of samples such as serum versus tissue, and still the specific dose relationship between Wnt inhibitors and bone formation is still unknown and current literature still lacks to establish normal reference ranges for those Wnt signaling inhibitors.

Age, Physical Activity, and Areal Bone Mineral Density

Based on animal and humans studies, it is now generally accepted that exercise improves bone mass and bone geometry and this effect is dependent on exercise intensity and duration. Physical activity during adolescence is associated with an increase in bone mass on the periosteal surfaces, while high impact and muscle loading exercise are associated with an increase in bone size. Therefore, peak bone mass, which is acquired during childhood and adolescence, could be the key determinant in lowering the risk of fracture in later life. Although peak bone mass is genetically determined, other determinants of peak bone mass are amenable to weight-bearing exercise and diet.

A significant main effect of physical activity was detected for total body and spine areal bone mineral density ($p<0.05$) and hip areal bone mineral density variables, and a main effect of age was detected for spine BMC ($p<0.05$). The present study also found a significant main effect of age and physical activity levels for left trochanter bone mineral density. The results of the current study are in line with the previous study by Liang et al. (2011), who found that after a 12-month strength training intervention there was a significant increase in heel areal bone mineral density in premenopausal women.

Similar to the present study, Baxter-Jones et al. (2008) reported that active adolescent females had significantly higher BMC and total femoral neck BMD compared to an inactive group, suggesting that participating in physical activity conserves skeletal formation benefits throughout the adult phase. Similarly, participating in high-impact exercise lead to a significant increase in femoral neck BMD in the exercise group compared to the control group that reflects the positive influence of high-impact exercise on bone (Bailey and Brooke-Wavell, 2010).

Accumulating evidence suggests that high-impact exercise has a direct impact on bone, as the strain is generated by contractile force from the tendon to the insertion site on bone leading to mechanical loading (Bailey and Brooke-Wavell, 2010; Mosti et al., 2014). Warren et al. (2008) showed a significant decrease in femoral neck BMD in their control group compared to a strength training group, which suggests the importance of mechanical loading for skeletal structure. In line with the present study, a randomized control trial found an increase in BMD and BMC at the lumbar spine in the training group compared to the control group (Mosti et al., 2014). Systemic reviews and

meta-analyses by Xu et al. (2016) reported that combined-impact exercise protocols have more significant effects on bone compared to short-bouts of exercise.

Both the intensity and frequency of exercise are considered as major determinants of bone response. In the present study, we did not measure intensity, frequency or total volume of exercise. The physical activity scores were based on a self-reported questionnaire. Although we found that total PA scores were higher in middle-aged women compare to young women, these values were not statistically significant. Terefore it is not possible to conclusively determine specific exercise effects on bone in the current study. A previous study by Bailey and Brooke-Wavell (2010) demonstrated that more frequent exercise is effective for bone adaptation, while the present study relied on the self-reported questionnaire for current and past BPAQ score.

Age, Physical Activity, and Volumetric Bone Mineral Density

In contrast to previous studies (Wilks et al., 2009; Evans et al., 2012), the current study did not find significant differences in bone geometry based on physical activity. The results showed a significant main effect of age on total volumetric BMC, trabecular BMC and BMD, and trabecular bone strength index at 4% tibia site ($p<0.05$). We also found a significant main effect of age at 38% cortical vBMD, 66% total area, periosteal circumference, and polar moment of inertia of the tibia. It is well documented that quantifying trabecular and cortical bone characteristics can identify the individual risk of fracture in later life (Hamilton et al., 2010). The results from the current study showed a trend that young women were taller than middle-aged women. Because middle-aged women were shorter, their smaller bones could be subjected to lower bone loading.

Increased periosteal growth is a key factor for protecting against bone loss during aging. It has been reported that bone resorption during aging occurs at the endocortical surface, and periosteal apposition needs to increase close to 350% so that gradual bone loss at the endosteal apposition is maintained (Jepsen and Andarawis-Puri, 2012). It has also been reported that regardless of physical activity, periosteal circumference increases with age (Kaji et al., 2005), which is in contrast with the current study.

The results from the current study showed a significant main effect of age on stress-strain index and polar moment of inertia. As mentioned in the literature, the polar moment of inertia reflects torsional rigidity and can better predict bone fracture than can non-directional geometric indicators (Augat et al., 1996), and the stress-strain index provides a good estimate of mechanical strength. Surprisingly, the current study showed that middle-aged women had significantly higher cortical vBMD at 38% tibia compared to young women. It has been well established that exercise primarily influences cortical bone parameters, particularly in cortical BMD, cortical area, and both distal and shaft sites (Liu-Ambrose et al., 2004 and Shedd et al., 2007). In the present cross-sectional study, we only measured physical activity scores, which were higher in middle-aged women, and although not significant could possibly have driven the result for cortical volumetric bone mineral density at the 38% tibia site.

Age, Physical Activity, and Hip Structural Analysis

In the current study, we also measured Hip Structural Analysis (HSA) parameters, another geometric measure and indicator of hip fracture risk at the femoral neck in women independent of BMD. According to Martin and Burr (1984), a one

standard deviation increase in HSA is associated with 1.8 fold increase in hip fracture risk. As mentioned previously, variations and bone strength differences that occur during aging are mostly geometric in nature, and accurate measurements could provide a proper assessment of the bone strength (Beck, 2007). The image we obtained from DXA is 2D but with the advancement in DXA software, we can assess bone geometry, which is helpful in determining fracture risk. The variables for HSA included in this study were SI, CSMI, SM, and BR (Faulkner et al., 2006; Bonnick, 1989; Beck, 2007, Roark and Young., 1989).

The results from the current study showed that there was a significant main effect of age on left SM, which is in line with Beck et al. (2000), that age is associated with a decline in sectional modulus at the proximal femur site. It has been reported that participating in high-impact exercise resulted in an increase in SM that disappears at 3.5 years (Heinonen et al., 2012). Although we did not find a significant main effect for physical activity in the current study, exercise or mechanical loading is associated with enhancing structural strength against compressive and bending loading (Heinonen et al., 2012). Further research on different loading modalities is necessary to understand skeletal geometric properties. One longitudinal study reported proximal femoral bone assessment with women spanning three generations including adolescence (girls), premenopausal (mothers) and postmenopausal women (maternal grandmothers) (Wang et al., 2014). They found that hip strength index decreased rapidly after menarche, remained constant afterward and significantly decreased after the postmenopausal stage.

The current study showed that there was a significant main effect of age and physical activity for right BR. A previous study reported that buckling ratio decreased

in the exercise group compared to the diet group in elderly people, which is in line with the current study, suggesting that mechanical loading plays an important role in elderly skeleton composition (Armamemento-Villareal et al., 2012) as it does in young women as shown in the current study. Notably, cortical thickness, lower femoral neck aBMD, and BR are strong predictors of cervical and trochanteric fracture, whereas CSMI and SI are only the determinants of trochanteric fracture (Szulc et al., 2006). One of the limitations of HSA is that the software could not determine periosteal and endosteal diameter that make difficult to explain the changes that occur in cross-sectional area (Wang et al., 2014).

Age, Physical Activity, and Muscular Function Assessment

The results from the current study on jump characteristics showed that, regardless of physical activity, there was a significant age effect for time in air, vertical jump height and relative jump power. There was also a significant age effect for 1RM muscle strength. Aging is associated with gradual loss of lean body mass and muscle function, including muscle power and strength (Janssen et al., 2000). Muscle power is considered a direct measure of muscle function and is positively associated with bone strength, especially in the cortical site (Hardcastle et al., 2014; Janz et al., 2015).

A previous cross-sectional study by Dietzel et al. (2013) reported that there was a 20% decline in force from the third to the ninth decade both in men and women. There was also 40-50% decline in power in women 40-49 years old. In contrast, the current study did not find any significant differences in power between two age groups; however, there was a significant age difference when power was expressed relative tp body weight (relative power).

One cross-sectional study reported more than 50% decline in peak power specific to body mass between the ages of 20-80 years (Runge et al., 2004). The authors also reported a correlation between jump power with age after adjusting for body mass, which is similar to the current study, where we reported significant differences in relative jump power. We also found significantly lower 1RM strength in middle-aged women compared to young women. The possible mechanisms for age-related decline in muscle strength include decreases in the size and number of fast twitch muscle fibers. The decrease in sliding speed of the myosin molecule and changes in the mechanical properties of tendons also exert an additive effect on age-related decline in muscle strength (Mosti et al., 2014; Narici et al., 2003).

It is well established that muscle contraction provides significant strain to stimulate osteogenic responses. Physical activity and intrinsic muscle properties are important factors to increase strain, which leads to bone adaptation. In support of this concept, high impact exercise such as the vertical jump has proven to be effective in improving bone health. The muscle cross-sectional area also plays an important role in muscle strength; however, in the current study, we did not find significant differences in the muscle cross-sectional area between two age groups.

Wnt Signaling Inhibitor Relationships with Bone Mineral Density, Hip Structural Analysis, and Muscle Strength Variables

The results from the bivariate correlations showed that both sclerostin and DKK-1 were weakly positively associated with spine BMD. Modder et al. (2010) and Sheng et al. (2011) reported that age-adjusted total body BMD, spine BMD and hip BMD were positively associated with sclerostin, which is in line with the current study.

Although the reason for this paradoxical association is still unknown, the weak positive relationship could be possibly due to two mechanisms; sclerostin is produced exclusively by osteocytes and could reflect the number of osteocytes, and increase in age leads to decrease in renal clearance of sclerostin.

In the current study, HEPA-active women had significantly higher sclerostin levels that lead to constant bone formation, reflecting an increase in the Wnt signaling pathway. This could possibly increase sclerostin levels so that bone homeostasis is maintained through a negative feedback signal on osteoblasts. In the current study, DKK-1 exhinited a weak positive relationship with spine BMD, which is in contrast to the previous studies by Hampson et al. (2013) who reported no association, and Butler et al. (2011) who reported a negative association. The weak positive association between the spine and hip BMD with DKK-1 levels could be partly explained by differences in tissue distribution (Hampson et al. 2013). Another explanation could be that DKK-1 is produced not only by the skeleton but also other tissues such as platelets and vascular cells (Thambiah et al., 2012). However, one study contradicted this explanation by showing a correlation between circulating sclerostin levels and marrow plasma levels, suggesting that circulating sclerostin levels do not reflect changes in bone cells (Drake et al., 2010). Therefore, weak positive correlation in the current study merits further investigation.

We did not find any association between sclerostin and DKK-1 levels with hip structural analysis variables, which is in contrast to the previous study by Armamento-Villareal et al. (2012), who reported significant correlation among sclerostin, sectional modulus, and buckling ratio. The possible explanation could be due to the age group, as

the previous study was done in elderly individuals where the buckling ratio was higher than for the premenopausal women in the current study.

There was a weak negative association for 38% cortical area, periosteal and endosteal circumference with sclerostin levels. However, sclerostin is moderately associated with cortical volumetric bone mineral density. It has been reported that SOST knockout mice have significantly higher cortical bone and mice with overexpressed SOST have thinner cortices and trabecular bone, reflecting sclerostin as a negative regulator in the skeleton (Li et al., 2008; Loots, et al., 2006). The moderate positive association might reflect osteocyte number, or could possibly difference in the secretion pattern in different skeletal tissue..

The moderate positive relationship between sclerostin levels and cortical vBMD in the current study is in line with a previous study (Thorson et al., 2013) that reported a positive relationship between sclerostin at 66% tibia, suggesting 66% tibia is comparatively less loaded than 38% tibia. From the perspective of strength, bone loading is highly dependent on mechanical forces rather than just gravitational loads. The weak negative association with 38% tibia site raises the possibility that sclerostin production regulates cortical structure, as higher loading leads to less sclerostin production. The current study did not find any significant relationships between DKK-1 and pQCT bone variables. In contrast to the current study, animal studies suggested that reduced DKK-1 expression is associated with significant increases in the proximal tibia, and overexpressed DKK-1 is associated with reduced proximal tibia density (MacDonald et al., 2007; Yao et al., 2011).

We also found weak negative relationships between vertical jump height and time in air with sclerostin levels. The previous study by Thorson et al. (2013) reported that sclerostin levels are negatively associated with grip strength, possibly due to mechanical forces caused by gravity. The inverse relationship from the current study suggests that changes in sclerostin levels could influence muscle activity, which could possibly increase serum sclerostin levels during muscle atrophy or disuse. A paradigm based on an animal study reported that Wnt7a signaling stimulated skeletal muscle growth and improved muscle strength via a non-canonical pathway through the activation JNK or AKT-mTOR (Bentzinger et al., 2014). However, due to lack in the literature, cross talk between muscle-bone and Wnt signaling pathway deserves further investigation.

To our knowledge, this is the first study to examine the relationship between sclerostin and DKK-1 levels with pQCT parameters in young and middle-aged women. To date, no studies have reported sclerostin and DKK-1 responses to muscle strength. The findings from the current study showed sclerostin and DKK-1 are important modulators of bone remodeling processes.

Although the current study had unique features, there are certain limitations. Since there are only a few studies of Wnt signaling inhibitors in humans, the results are largely explained based on the animal studies. In humans, it is not feasible to examine changes in osteocyte expression of sclerostin in bone. In this cross-sectional study, a self-reported IPAQ questionnaire was used to calculate physical activity scores, which could have influenced the results. Also, we did not measure bone formation, resorption, serum calcium, PTH, IGF-1, or serum vitamin D levels that could have prevented in-

depth understanding of Wnt signaling inhibitors and bone characteristics in premenopausal women.

The current study measured serum sclerostin concentrations ranging from 0.27 to 0.91 ng/ml and serum DKK-1 concentrations ranging from 15.53 to 86.16 pmol/l. The current study utilized TECO and Eagle manufacturer kits for sclerostin and DKK-1 assays, respectively. It has been reported that different commercially available sclerostin assay kits detect large differences in the sclerostin levels. Therefore, standardization of the assays should be done cautiously with attention to reference ranges to use sclerostin and DKK-1 as the diagnostic tool for measuring bone diseases (Costa et al., 2017; Clarke and Drake, 2013).

Chapter 5: Conclusions

The primary purpose of this study was to compare serum concentrations of sclerostin and DKK-1 in young (20-30 years) and middle-aged (35-45 years) premenopausal women. These age groups were selected to allow comparisons between women who are still accruing bone mass versus those who have already achieved their peak bone mass. The following research questions were investigated: 1. Will there be a significant difference in sclerostin and DKK-1 serum concentrations between two age groups of young (20-30 years) and middle-aged premenopausal women (35-45 years)? 2. Will there be a significant difference in sclerostin and DKK-1 serum concentrations based on the physical activity status (low, moderate, and HEPA-active women)? 3. Will there be a significant interaction between age groups (20-30 years and 35-45 years) and physical activity status (low, moderate, and HEPA-active women) for sclerostin and DKK-1 serum concentrations?

Hypothesis 1. Sclerostin and DKK-1 serum concentrations will be lower in young women (20-30 years) compared to middle-aged premenopausal women (35-45 years).

Yes, findings from the current study showed sclerostin levels were significantly lower in young women compared to middle-aged women ($p<0.001$). We did not find significant age group differences for DKK-1 levels.

Hypothesis 2. Women with higher physical activity levels will have lower sclerostin and DKK-1 serum concentrations.

Findings from the current study do not support this hypothesis. Sclerostin levels were significantly higher in HEPA-active women compared to low-moderately active women ($p<0.05$). We did not find significant differences based on physical activity levels for DKK-1 concentrations.

Hypothesis 3. There will be no interaction between age and activity level, as both age groups will show the same pattern for sclerostin and DKK-1 serum concentrations, which will decrease as physical activity level (low-moderate and HEPA-active) increases.

The findings from this study showed significant main effects of age or physical activity only for sclerostin. There was no significant age group × physical activity status interaction for either sclerostin or DKK-1.

Sub-hypothesis 1. There will be a moderate inverse relationship between sclerostin and DKK-1 serum concentrations and aBMD and vBMD in young and middle-aged premenopausal women.

This hypothesis was not supported by the findings of this study. Sclerostin showed a significant low positive relationship with hip aBMD variables, specifically right neck Z-Score, left neck Z-Score, right trochanter BMD, right trochanter Z-Score, left trochanter Z-score and total right BMD. DKK-1 also showed a low positive relationship with right and left trochanter BMD, and right and left trochanter Z-Score.

There were no significant correlations between sclerostin and DKK-1 levels and hip structural analysis variables (strength index, buckling ratio, sectional modulus, and CSMI). There were no correlations observed for sclerostin and DKK-1 levels with 4% pQCT variables. There was a significant low negative correlation between sclerostin

and total area, periosteal circumference, and endosteal circumference, a significant low positive correlation for total vBMD, and a moderate positive relationship for cortical vBMD at 38% tibia. However, no significant correlations were observed between DKK-1 and 38% pQCT variables. There was a significant low positive relationship between sclerostin and cortical vBMD at 66% tibia. However, there was no significant association found between DKK-1 and 66% tibia variables.

Sub-hypothesis 2. There will be a strong inverse relationship between sclerostin and DKK-1 serum concentrations with jump power and leg press in young and middle-aged premenopausal women.

There was significant low negative association for time in air and vertical jump height variables with sclerostin levels. However, DKK-1 did not show any significant correlation with muscle strength variables.

Clinical Significance

The evidence on Wnt signaling and bone formation provide critical cues for the treatment of skeletal disorders such as osteoporosis. Sclerostin and DKK-1 are potent regulators of the Wnt signaling pathway, and directly bind to Wnt ligands and degrade β-catenin before it translocates into the nucleus and suppresses gene transcription for bone formation. According to the International Osteoporosis Foundation and International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) recommendation, procollagen type I N-terminal peptide (P1NP) and C-terminal telopeptide of type I collagen (CTx), should be used in predicting osteoporosis (Vasikaran et al., 2011). Notably, these markers reflect the functions of osteoblasts and osteoclasts and not the osteocytes. The Wnt signaling inhibitors sclerostin and DKK-1

are exclusively produced and secreted by osteocytes and act as negative modulators of bone metabolism. Therefore, these markers could be used as fracture predictors of osteoporosis both in men and women.

Sclerostin and DKK-1 provide an opportunity for developing new anabolic therapies, including anti-sclerostin and anti-DKK-1 antibodies that increase rapid fracture healing and bone mineral density. These therapies could provide a promising approach in treating osteoporosis and skeletal disorders. Recent results from phase three clinical trials for the drug romosozumab, showed a significant reduction in vertebral and clinical fracture in osteoporotic postmenopausal women (Cosman et al., 2016). Similarly, use of a bispecific antibody against sclerostin and DKK-1 has shown synergistic bone formation and fracture healing in mice and non-human primates (Florio et al., 2016). Therefore, understanding the regulation of SOST in human osteoblastic cell lines and epigenetic modification could be useful in identifying an additional target to treat bone diseases.

Future Directions

As mentioned previously, sclerostin is exclusively secreted from osteocytes and has provided an important step in understanding the regulation of bone remodeling. Animal studies have reported that sclerostin and DKK-1 expression was downregulated in the presence of mechanical loading. Studies on different types of exercise such as resistance exercise, jump exercise, whole-body vibration, and endurance exercise are limited in humans and deserve further attention. Therefore, future research in humans should also include developing exercise interventions for sclerostin and DKK-1 along with changes in BMD and bone strength parameters in both genders with different age

groups and with large sample size. The measurement of Wnt signaling inhibitors is novel to bone research and this technology still continues to advance. Therefore, standardized procedures for serum assays should be incorporated so that reliable reference ranges of sclerostin and DKK-1 can be established. Long-term exercise interventions can detect changes in BMD compared to short-term studies, therefore, future studies should involve long-duration exercise interventions in comparing sclerostin and DKK-1 levels. It is widely accepted that exercise has a positive impact on bone, incorporating exercise responses in Wnt pathway could explore avenues in bone biology. Further, study on vitamin D, calcium, and parathyroid hormones analysis along with Wnt signaling inhibitors in intervention studies could provide knowledge in the biology of bone and Wnt signaling pathway.

Sclerostin and DKK-1 are secreted at high levels during unloading conditions such as bed rest, and astronauts during space missions. The lack of weight-bearing forces exerts a negative effect on bone cells in the microgravity environment due to there being less mechanical strain. Microgravity induces muscle atrophy and bone loss that can deteriorate astronauts' health and performance. Therefore, understanding the role of sclerostin and DKK-1 in microgravity could contribute in developing a strategy for space travel and for a future human mission to Mars.

In summary, both human and animals showed Wnt signaling as a negative regulator of bone mass and potential concerns regarding mode of action to enhance bone repair along with exercise both in men and women deserves further investigation.

References

Agholme F, Aspenberg P. 2011 Wnt signaling, and orthopedics, an overview. Acta Orthop. 82(2); 125-130.

Alemeida M, Han L, Martin-Millan M, O'Brien CA, Manolagas SC. 2007 Oxidative stress antagonizes Wnt signaling in osteoblast precursors by diverting beta-catenin from T cell factor to forkhead box O-mediated transcription. J Biol Chem. 282(37); 27298-27305.

Almeida M, Han L, Bellido T, Manolagas SC, Kousteni S. 2005 Wnt proteins prevent apoptosis of both uncommitted ostehuioblast progenitors and differentiated osteoblasts by beta-catenin-dependent and independent signaling cascades involving Src/ERK and phosphatidylinositol 3-kinase/AKT. J Biol Chem. 280; 41342-41351.

Amrein K, Amrein S, Drexler C, Dimai HP, Dobnig H, Pfeifer K, et al. 2011 Sclerostin and its association with physical activity, age, gender, body composition, and bone mineral content in healthy adults. J Clin Endocr Metab. 97(1):148-54.

Anliker E, Dick C, Rawer R, Toigo M. 2012 Effects of jumping exercise on maximum ground reaction force and bone in 8 to 12-year-old boys and girls: a 9-month randomized controlled trial. J Musculoskelet Neuronal Interact. 12 (2); 56-57.

Ardawi MS, Al-Kadi HA, Rouzi AA, Qari MH. 2011 Determinants of serum sclerostin in healthy pre- and postmenopausal women. J Bone Miner Res. 26(12):2812-22.

Ardawi MS, Rouzi AA, Qari MH. 2012 Physical activity in relation to serum sclerostin, insulin-like growth factor-1, and bone turnover markers in healthy premenopausal women: a cross-sectional and a longitudinal study. J Clin Endocr Metab. 97 (10):3691-3699.

Armamento-Villareal R, Sadler C, Napoli N, Shah K, Chode S, Sinacore DR, et al. 2012 Weight loss in obese older adults increases serum sclerostin and impairs hip geometry but both are prevented by exercise training. J Bone Miner Res. 27(5):1215-1221.

Arnaud CD. 1996 Osteoporosis: using bone markers for diagnosis and monitoring. Geriatrics. 51: 24-36.

Bailey CA, Brooke-Wavell K. 2010 Optimum frequency of exercise for bone health: randomized controlled trial of a high-impact unilateral intervention. Bone 26(4): 1043–1049.

Baran DT. 1999 New ways to manage the old problem of osteoporosis. JAAPA. 12:42-52.

Baxter-Jones AD, Kontulainen SA, Faulkner RA, Bailey DA. 2008 A longitudinal study of the relationship of physical activity to bone mineral accrual from adolescence to young adulthood. Bone. 43(6):1101-1107.

Beck TJ. 2007 Extending DXA beyond bone mineral density; understanding hip structural analysis. Curr Osteoporos Rep. 5 (2); 49-55.

Beck TJ, Looker AC, Ruff CB, Sievanen H, Wahner HW. 2000 Structural trends in the aging femoral neck and proximal shaft: analysis of the Third National Health and Nutrition Examination Survey dual-energy X-ray absorptiometry data. J Bone Miner Res. 15: 2297-2304.

Bell A. 2008 The pipe and the pinwheel: is pressure an effective stimulus for the 9+0 primary cilium? Cell Bio Int. 32; 462-468.

Bellido T, Plotkin LI, Bruzzaniti A. 2014 Bone morphology and organization. In: Burr DB, Allen MR (Ed). Basic and applied bone biology. Academic Press. PP. 27-45.

Bentzinger CF, von Maltzahn J, Dumont NA, Stark DA, Wang YX, Nhan K, et al. 2014 Wnt7a stimulates myogenic stem cell motility and engraftment resulting in improved muscle strength. J Cell Biol 205:97–111.

Berger C, Goltzman D, Langsetmo L, et al. 2010 Peak bone mass from longitudinal data: implications for the prevalence, pathophysiology, and diagnosis of osteoporosis. J Bone Miner Res. 25(9): 1948-1957.

Biam S, Binkley N, Bilezikian JP. 2008 Official positions of the international society for clinical densitometry and executive summary of the 2007 ISCD position development conference. J Clin Densitom. 11(1): 75-91.

Blake G, Adams JE, Bishop N. 2013 DXA in adults and children. In: Rosen CJ (Ed). Primer on the metabolic bone diseases and disorders of mineral metabolism. John Wiley and Sons. pp. 251-263.

Bonewald LF. 2011 The amazing osteocyte. J Bone Miner Res. 26(2):229-238.

Bonewald LF. 2013 Osteocytes. In: Rosen CJ (Ed). Primer on the metabolic bone diseases and disorders of mineral metabolism. John Wiley and Sons. PP. 34-39.

Bonnick SL. 2007 HSA: beyond BMD with DXA. Bone. 41; 1 Suppl 1:S9-12.

Boskey AL, Gadaleta S, Gundberg C, Doty SB, Ducy P, Karsenty G. 1998 Fourier transform infrared microspectroscopic analysis of bones of osteocalcin-deficient mice provides insight into the function of osteoclacin. Bone. 23; 187-196.

Bourrin S, Ghaemmaghami F, Vico L, Chappard D, Gharib C, Alexandre C. 1992 Effect of a five-week swimming program on rat bone: a histomorphometric study. Calcif Tissue Int. 51(2); 137–42.

Bouxein ML. 2008 Technology Insight: noninvasive assessment of bone strength in osteoporosis. Nature. 4(6):310-318.

Buehring B, Krueger D, Binkley N. 2010 Jumping mechanography: a potential tool for sarcopenia evaluation in older individuals. J Clinical Densitom. 13(3): 283-291.

Burr DB, Akkus O. 2014 Bone morphology and organization. In: Burr DB, Allen MR (Ed). Basic and applied bone biology. Academic Press. PP. 1-24.

Butler JS, Murray DW, Hurson CJ, O'Brien J, Doran PP, O'Byrne JM. 2011 The role of Dkk1 in bone mass regulation: correlating serum Dkk1 expression with bone mineral density. J Orthop Res. 29:414-8.

Chen Y, Whetstone HC, Youn A, et al. 2007 Beta-catenin signaling pathway is crucial for bone morphogenetic protein 2 to induce new bone formation. J Biol Chem. 282:526-533.

Clarke BL, Khosla S. Female reproductive system and bone. 2010 Arch Biochem Biophys. 503(1):118-28.
Clevers H, Nusse XR. 2012 Wnt/beta-catenin signaling and disease. Cell. 149:1192–205.

Cohen J. 1992 Statistical power analysis. Current Dir Psychol Sci. 1(3) 98-101.

Cointry GR, Ferretti JL, Reina PS, et al. 2014 The pQCT Bone strength indices (BSIs, SSI). Relative mechanical impact and diagnostic value of the indicators of bone tissue and design quality employed in their calculation in healthy men and pre- and post-menopausal women. J Musculoskelet Neuronal Interact. 14(1): 29-40.

Cooper C, Campion G, Melton LJ. 1992 Hip fractures in the elderly: a world-wide projection. Osteoporos Int. 2 (6): 285-289.

Cooper, A, and Cooper B.B. 1822 A treatise on dislocations, and on fractures of the joints. Churchill. London, United Kingdom. 425 pp.

Cosman F, Crittenden DB, Adachi JD, Binkley N, Czerwinski E. et al. 2016 Romosozumab treatment in postmenopausal women with osteoporosis. N Engl J Med. 375: 1532–1543.

Costa AG, Cremers S, Bilezikian JP. 2017 Sclerostin measurement in humans: validity and current limitations. Bone. 96: 24-28.

Cox LG, van Rietbergen B, van Donkelaar CC, Lto K. 2011 Analysis of bone architecture sensitivity for changes in mechanical loading, cellular activity, mechanotransduction, and tissue properties. Biomech Modeling Mechanobiol. 10(5); 701-712.

Craig Cl, Marshall AL, Sjostrom M, Bauman AE, Booth ML, Ainsworth BE, et al. 2003 International physical activity questionnaire: 12-country reliability and validity. Med Sci Sports Exerc. 35(8): 1381-95.

Cui Y, Niziolek PJ, macDonald BT, Zylstra CR, Alenina N, Robinson DR, et al. 2011 Lrp5 functions in bone to regulate bone mass. Nat Med. 17; 684-691.

de Gorter DJJ, ten Dijke P. 2013 Signal transduction cascades controlling osteoblast differentiation. In: Rosen CJ (Ed). Primer on the metabolic bone diseases and disorders of mineral metabolism. John Wiley and Sons. PP. 15-33.

Diarra D, Stolina M, Polzer K, et al. 2007 Dickkopf-1 is a master regulator of joint remodeling. Nat Med. 13 (2):156-163.

Dietzel R, Gast U, Heine T, et al. 2010 Cross-sectional assessment of neuromuscular function using mechanography in women and men aged 20–85 years. J Musculoskelet Neuronal Interact. 13(3):312–319.

Drake MT, Srinivasan B, Modder UI, Peterson JM, McCready LK, Riggs BL, et al. 2010 Effects of parathyroid hormone treatment on circulating sclerostin levels in postmenopausal women. J Clin Endocrinol Metab. 95(11):5056–62.

Eriksen EF. 2010 Cellular mechanisms of bone remodeling. Rev Endocr Metab Disord. 11; 219-227.

Faul F, Erdfelder E, Lang AG, Buchner A. 2007 G* Power 3: A flexible statistical power analysis program for the social, behavioral, and biomedical sciences. Behav Res Meth. 39(2):175-91.

Faulkner KG, Wacker WK, Barden HS, Simonelli C, Burke PK, Ragi S, et al. 2006 Femur strength index predicts hip fracture independent of bone density and hip axis length. Osteoporos Int. 17(4):593-9.

Frings-Meuthen P, Boehme G, Liphardt AM, Baecker N, Heer M, Rittweger J. 2013 Sclerostin and DKK1 levels during 14 and 21 days of bed rest in healthy young men. J Musculoskelet Neuronal Interact. 13(1):45-52.

Florio M, Gunasekaran K, Stolina M. et al. 2016 A bispecific antibody targeting sclerostin and DKK-1 promotes bone mass accrual and fracture repair. Nat Commun. 7: 11505.

Frost HM. 2000 Muscle, bone, and the Utah paradigm: a 1999 overview. Med Sci Sports Exerc. 2(5); 911-917.

Frost HM. 2001 From Wolff's Law to the Utah Paradigm: insights about bone physiology and its clinical applications. Anat Rec. 262: 398-419.

Galea GL, Sunters A, Meakin LB, Zaman G, Sugiama T, Lanyon LE. 2011 SOST down-regulation by mechanical strain in human osteoblastic cells involves PGE2 signaling via EP4. FEBS Lett. 585; 2450-2454.

Gardinier JD, Townend CW, Jen KP, Wu Q, Duncan RL, Wang L. 2010 In situ permeability measurement of the mammalian lacunar-canalicular system. Bone. 46 (4); 1075-81.

Garnero P, Sornay-Rendu E, Munoz F, Borel O, Chapurlat RD. 2013 Association of serum sclerostin with bone mineral density, bone turnover, steroid and parathyroid hormones, and fracture risk in postmenopausal women: the OFELY study. Osteoporos Int. 24(2):489-94.

Gaudio A, Pennisi P, Bratengeier C, Torrisi V, Linder B, Mangiafico RA. 2013 increased sclerostin serum levels associated with bone formation and resorption markets in patients with immobilization-induced bone loss. J Clin Endocr Metab. 95(5).

Grasso D, Corsetti R, Lanteri P, Di Bernardo C, Colombini A, et al. 2015 Bone-muscle unit activity, salivary steroid hormones profile, and physical effort over a 3-week stage race. Scand J Med Sci Sports 25(1): 70-80.

Gusi N, Raimundo A, Leal A. 2006 Low-frequency vibratory exercise reduces the risk of bone fracture more than walking. A randomized controlled trial. BMC Musculoskel Disord. 7(1): 92.

Hagstromer M, Oja P, Sjostrom M. 2006 The international physical activity questionnaire (IPAQ): a study of concurrent and construct validity. Public Health Nutrition. 9(06): 755-762.

Hamilton CJ, Swan VJD, Jamal SA. 2010 The effects of exercise and physical activity participation of bone mass and geometry in postmenopausal women: a systematic review of pQCT studies. Osteoporos Int. 21(1):11-23.

Han Y, Cowin SC, Schaffler MB, Weinbaum S. 2004 Mechantransduction and strain amplification in osteocyte cell process. Proc Natl Acad Sci USA. 101(47); 16689-16694.

Hardcastle SA, Gregson CL, Rittweger J, Crabtree N, Ward K, and Tobias JH. 2014 Jump power and force have distinct association with cortical bone parameters: findings from a population enriched by individuals with high bone mass. J Clin Endocrinol Metab. 99(1): 266-275.

Harter CS, Bouxein M, Lewis B, et al. 1990 Muscle strength as a predictor of bone mineral density in young women. J Bone Min Res. 5(6): 589-595.

Hay E, Faucheu C, Suc-Royer I, Touitou R, Stiot V, Vayssiere B, et al. 2005 Interaction between Lrp5 and Frat 1 mediates the activation of the Wnt canonical pathway. J Biol Chem. 280; 13616-13623.

Heionen A, Sievanen H, Kyrolainen H, Pertunen J, Kannus P. 2001 Mineral mass, size, and estimated mechanical strength of triple jumpers' lower limb. Bone. 29(3):279-285.

Heinonen A, Mantynen J, Kannus P, Uusi-Rasi K, Nikander R et al. 2012 Effects of high-impact and detraining on femoral neck structure in premenopausal women: a hip structural analysis of 18-month randomized controlled exercise intervention with 3.5 year follow-up. Physiother Can. 64 (1): 98-105.

Hinton PS, Nigh P, Thyfault J. 2017 Serum sclerostin decreases following 12 months of resistance-or jump-training in men with low bone mass. Bone. 96: 85-90.

Hind K, Burrowa M. 2007 Weight-bearing exercise and bone mineral accrual in children and adolescents: A review of controlled trials. Bone. 40(1): 14-27.

Huiskes R, Ruimerman R, van Lenthe GH, Janssen JD. 2000 Effect of mechanical forces on maintenance and adaptation of form in trabecular bone. Nature. 405(6787); 704-706.

Ingber DE. 2005 Mechanical control of tissue growth: function follows form. Proc Natl Acad Sci USA. 102(33); 11571-11572.

Janssen I, Heymsfield SB, Wang ZM, Ross R. 2000 Skeletal muscle mass and distribution in 468 men and women aged 18-88 yr. J Appl Physiol. 89(1):81-88.

Janz, K. F., Letuchy, E. M., Burns, T. L., Francis, S. L., & Levy, S. M. (2015). Muscle power predicts adolescent bone strength: Iowa bone development study. Med Sci Sports Exercise. 47(10), 2201-2206.

Jepsen KJ, Andarawis-Puri N. 2012 The amount of periosteal apposition required to maintain bone strength during aging depends on adult bone morphology and tissue-modulus degradation rate. J Bone Miner Res 27(9): 1916-1926.

Ju Y-I, Sone T, Ohnaru K, Choi H-J, Fukunaga M. 2012 Differential effects of jump versus running exercise on trabecular architecture during remobilization after suspension-induced osteopenia in growing rats. J Appl Physiol. 112(5); 766–772.

Kaji H, Kosaka R, Yamauchi M, Kuno K, Chihara K, Sugimoto T. 2005 Effects of age, grip strength and smoking on forearm volumetric bone mineral density and bone geometry by peripheral quantitative computed tomography: comparisons between female and male. Endocr J. 52(6): 659–666.

Kamel MA, Picconi JL, Lara-Castillo N, Johnson ML. 2010 Activation of β-catenin signaling in MLO-Y4 osteocytic cells versus 2T3 osteoblastic cells by fluid flow shear stress and PGE2: implications for the study of mechanosensation in bone. Bone. 47(5); 872-881.

Kang KS, Robling AG. 2015 New insights into Wnt-Lrp5/6-β-catenin signaling in mechanotransduction. Front Endocrinol. 5:246.

Kanis JA, Melton LJ. Christiansen C, Johnston CC, Khaltaev N. 1994 The diagnosis of osteoporosis. J Bone Miner Res. 9(8): 1137-1141.

Kato M, Patel MS, Levasseur R, Lobov I, Chang BHJ, Glass II DA, et al. 2002 Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. J Cell Biol. 157(2): 303-314.

Kato T, Niwa M, Yamashita T, Matumoto M, Umemura Y. 2015 Past sporting activity during growth induces greater bone mineral content and enhances bone geometry in young men and women. J Bone Miner Metab. 33(5); 569-576.

Kelley GA, Kelley KS, Kohrt WM. 2013 Exercise and bone mineral density in premenopausal women: A meta-analysis of randomized controlled trials. Intl J Endocrinol. 2013:741639.

Kerschan-Schindl K, Thalmann MM, Weiss E, Tsironi M, Föger-Samwald U, Meinhart J, et al. 2015 Changes in serum levels of myokines and Wnt-antagonists after an ultramarathon race. PloS One. 10(7):e0132478.

Khosla S. 2013 Pathogenesis of age-related bone loss in humans. J Gerontol Biol Sci Med Sci. 68(10): 1226-1235.

Kikuchi A, Yamamoto H, Sato A. 2009 Selective activation mechanisms of Wnt signaling pathways. Trends Cell Biol. 19(3):119–29.

Kim TH, Chang JS, Park KS, Park J, Kim N, Lee JI, et al. 2017 Effects of exercise training on circulating levels of Dickkpof-1 and secreted frizzled-related protein-1 in breast cancer survivors: A pilot single-blind randomized controlled trial. PLoS One. 12(2): e0171771.

Kinsley MA, Semevolos SA, Duesterdieck-Zellmer KF. 2015 Wnt/beta catenin signaling of cartilage canal and osteochondral junction chondrocytes and full thickness cartilage in early equine osteochondrosis. J Orthopaed Res. 33(10): 1433-1438.

Kishimoto K, Lynch RP, Reiger J, Yingling VR. 2012 Short-term jump activity on bone metabolism in female college-aged non-athletes. J Sports Sci Med. 11(1):31-38.

Klein-Nulend J, Bakker AD, Bacabac RG, Vatsa A, Weinbaum S. 2013 Mechanosensation and transduction in osteocytes. Bone. 54; 182-190.

Kovárová J, Hamar D, Sedliak M, Cvečka J, Schickhofer P, Böhmerová Ľ. 2015 Acute response of bone metabolism to various resistance exercises in women. Acta Facultatis Educationis Physicae Universitatis Comenianae. 55(1):11-19.

Kramer I, Halleux C, Keller H, Pegurri M, Gooi JH, Weber PB, et al. 2010 Osteocyte Wnt/beta-catenin signaling is required for the normal bone homeostasis. Moll Cell Biol. 30(12); 3071-3085.

Kremer R, Campbell P, Reinhardt T, Gilsanz V. 2009 Vitamin D status and its relationship to body fat, final height, and peak bone mass in young women. J Clin Endocrinol Metab. 94(1): 67-73.

Kronenberg H, Kobayashi T. 2004. Transcriptional regulation in development of bone. Endocrinol. 146(3):1012–1017.

Lane JM, Serota AC, Raphael B. 2006 Osteoporosis: Differences and similarities in male and female patients. Orthop Clin N Am. 37(4); 601–609.

Li C, Xing Q, Yu B, Xie H, Wang W, Shi C, et al. 2013 Disruption of LRP6 in osteoblasts blunts the bone anabolic activity of PTH. J Bone Miner Res. 28 (10); 2094-2108.

Li J, Sarosi I, Cattley RC, Pretorius J, Asuncion F, Grisanti M, et al. 2006 Dkk1-mediated inhibition of Wnt signaling in bone results in osteopenia. Bone 39(4):754 –766.

Li X, Ominsky MS, Warmington KS, et al. 2009 Sclerostin antibody treatment increases bone formation, bone mass, and bone strength in a rat model of postmenopausal osteoporosis. J Bone Miner Res. 24(4); 578-588.

Liang MTC, Braun W, Bassin SL, et al. 2011 Effect of high impact aerobics and strength training on BMD in young women aged 20-35 years. J Sports Med. 32(2):100–108.

Liu-Ambrose TY, Khan KM, Eng JJ, et al. 2004 Both resistance and agility training increase cortical bone density in 75- to 85- year-old women with low bone mass: a 6-month randomized controlled trial. J Clin Densitom 7 (4):390–398.

Liu LJ, Maruno R, Mashimo T, Sanka K, Higuchi T, Hayashi K, et al. 2003 Effects of physical training on cortical bone at midtibia by peripheral QCT. J Appl Physiol. 95(1): 219-224.

Logan CY, Nusse R. 2004 The Wnt signaling pathway in development and disease. Annu Rev Cell Dev Biol. 20; 781-810.

Loots GG, Kneissel M, Keller H, Baptist M, Chang J, Collette NM, Ovcharenko D, Plajzer-Frick I, Rubin EM. 2005 Genomic deletion of a long-range bone enhancer misregulates sclerostin in Van Buchem disease. Genome Res 15(7): 928 –935.

Lombardi G, Lanteri P, Colombini A, Banfi G. 2012 Blood biochemical markers of turnover: pre-analytical and technical aspects of sample collection and handling. Clin Chem Lab Med. 50(5); 771-789.

Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. 2013 The hallmarks of aging. Cell. 153(6); 1194-1217.

Macias BR, Swift JM, Nilsson MI, Hogan HA, Bouse SD, Bloomfield, SA. 2012 Simulated resistance training, but not alendronate, increases cortical bone formation and suppresses sclerostin during disuse. J Appl Physiol 112(5): 918–925.

MacDonald BT, Joiner DM, Oyserman SM, Sharma P, Goldstein SA, He X, Hauschka PV 2007 Bone mass is inversely proportional to Dkk1 levels in mice. Bone 41(3):331– 339.

Manolagas SC, Almeida M. 2007 Gone with Wnts: beta-catenin, T-cell factor, forkhead box O, and oxidative stress in the age-dependent diseases of bone, lipid, and glucose metabolism. Mol Endocrinol. 21(11); 2605-2614.

Mao B, Wu W, Davidson G, Marhold J, Li M, Mechler BM, et al. 2002 Kremen proteins are Dickkopf receptors that regulate Wnt/β-catenin signaling. Nature. 417(6889); 664-667.

Meakin LB, Price JS, Lanyon LE. 2014 The contribution of experimental in vivo models to understanding the mechanisms of adaptation to mechanical loading in bone. Front Endocrinol. 5: 154. doi:10.3389/fendo.2014.00154.

Mirza FS, Padhi ID, Raisz LG, Lorenzo JA. 2010 Serum sclerostin levels negatively correlate with parathyroid hormone levels and free estrogen index in postmenopausal women. J Clin Endocr Metab. 95(4):1991-1997.

Mödder UI, Hoey KA, Amin S, McCready LK, Achenbach SJ, Lawrence Riggs B, Joseph Melton L 3rd, Khosla S. 2011 Relation of age, gender, and bone mass to circulating sclerostin levels in women and men. J Bone Miner Res 26(2):373–379.

Moon RT, Kohn AD, De Ferrari GV, Kaykas A. 2004 Wnt beta-catenin signaling: diseases and therapies. Nat Rev Genet. 5(9); 691-701.

Morgan EF, Barnes GL, Einhorn TA. 2013 In: Marcus R, Feldman D, Dempster DW, Luckey M, Cauley J (Ed). The bone organ system: form and Function. Academic Press. Osteoporosis. pp. 3-20.

Morvan F, Boulukos K, Clement-Lacroix P, Roman Roman S, Suc-Royer I, Vayssiere B, Ammann P, et al. 2006 Deletion of a single allele of the Dkk1 gene leads to an increase in bone formation and bone mass. J Bone Miner Res 21(6):934 –945.

Mosti MP, Carlsen T, Aas E, Hoff J, Stunes AK, Syversen U. 2014 Maximal strength training improves bone mineral density and neuromuscular performance in young adult women. J Strength Cond Res. 28(10):2935-2945.

Musgrave KO, Giambalvo L, Leclerc HL, Cook RA. 1989 Validation of a quantitative food frequency questionnaire for rapid assessment of dietary calcium intake. J Am Diet Assoc. 89(10):1484– 1488.

Narici MV, Maganaris CN, Reeves ND, Capadoglio P. 2003 Effects of aging on human muscle architecture. J Appl Physiol. 95(6): 2229-2234.

Nattiv A, Loucks AB, Manore MM, et al. 2007 American College of Sports Medicine position stand. The female athlete triad. Med Sci Sports Exerc. 39 (10): 1867-1882.

Nilsson M, Ohlsson C, Mellstrom D, Lorentson M. 2009 Previous sport activity during childhood and adolescence is associated with increased cortical bone size in young adult men. J Bone Miner Res. 24 (1): 125-133.

Ozcivivi E, Luu YK, Adler B, Qin YX, Rubin J, Judex S, et al. 2010. Mechanical signals as anabolic agents in bone. Nature Rev Rheumatol. 6(1); 50-59.

Price C, Zhou X, Li W, Wang L. 2011 Real-time measurement of solute transport within the lacuna-canalicular system of mechanically loaded bone: direct evidence for load-induced fluid flow. J Bone Miner Res. 26(2); 277-285.

Reya T, Clevers H. 2005 Wnt signaling in stem cells and cancer. Nature. 434(7035); 843-850.

Riddle RC, Diegel CR, Leslie JM, Van koevering KK, Faugere MC, Clemens TL, et al., 2013 Lrp5 and Lrp6 exert overlapping functions in osteoblasts during postnatal bone acquisition. PloS One. 8(5); e63323.

Roark RJ, Young WC. 1989 Roark's formulas for stress and strain. 6th ed. New York: McGraw-Hill; 1989. p. 688.

Robling AG, Bellido T, Turner CH. 2006 Mechanical stimulation in vivo reduces osteocyte expression of sclerostin. J Musculoskel Neur Interact. 6(4); 354.

Robling AG, Burr DB, Turner CH. 2001 Recovery periods restore mechanosensitivity to dynamically loaded bone. J Exp Biol. 204(19); 3389–3399.

Robling AG, Castillo AB, Turner CH. 2006 Biomechanical and molecular regulation of bone remodeling. Annu Rev Biomed Eng. 8:455-98.

Roodman GD. 2006 New potential targets for treating myeloma bone disease. Clin Cancer Res 12(20):6270s– 6273s.

Roux W. 1881 Gessammelte abhandlungen. Leipzig, Engelmann. Bd. I pp 102-104.

Runge M, Rittweger J, Russo CR, Schiessl H, Felsenberg D. 2004 Is muscle power output a key factor in the age-related decline in physical performance? A comparison of muscle cross section, chair-rising test and jumping power. Clin Physiol Funct Imaging. 24(6): 335-340.

Salih DA, Brunet A. 2008 FoxO transcription factors in the maintenance of cellular homeostasis during aging. Curr Opin Cell Biol. 20(2): 126-136.

Salo J, Lehenkari P, Mulari M, Metsikkö K, Väänänen HK. 1997 Removal of osteoclast bone resorption products by transcytosis. Science. 276(5310):270-3.

Santos A, Bakker AD, Zandieh-Doulabi B, de Blieck-Hogervorst JM, Klein-Nulend J. 2010 Early activation of the beta-catenin pathway in osteocytes is mediated by nitric oxide, phosphatidyl inositol-3 kinase/Akt, and focal adhesion kinase. Biochem Biophys Res Commun. 391(1); 364-369.

Santos A, Bakker AD, Zandieh-Doulabi B, Semeins CM, Klein-Nulend J. 2009 Pulsating fluid flow modulates gene expression of Wnt signaling pathway in osteocytes. J Orthop Res. 27(10); 1280-1287.

Sawakami K, Robling AG, Ai M, Pinter ND, Liu D, Warden Sj, et al. 2006 The Wnt co-receptor LRP5 is essential for skeletal mechanotransduction but not for the anabolic bone response to parathyroid hormone treatment. J Biol Chem. 281(33); 23698-23711.

Sherk VD, Bemben MG, Bemben DA. 2010 Comparison of bone mineral density and bone quality in adult rock climbers, resistance-trained and untrained men. J Strength Cond Res. 24(9): 2468-2474.

Silva MJ, Gibson LJ. 1997 Modeling the mechanical behavior of vertebral trabecular bone: effects of age-related changes in microstructure. Bone. 21 (2) 191-1999.

Slinde F, Suber L, Edwen CE, Svantesson U. 2008 Test-retest reliability of three different countermovement jumping tests. J Strength Cond Res. 22(2): 640-644.

Spyropoulou A, Karamesinis K, Basdra E. 2015 Mechanotransduction pathways in bone pathobiology. Biochim Biophys Acta. 1852(9); 1700-1708.

Stenbeck G. 2002 Formation and function of the ruffled border in osteoclasts. Seminars Cell Devel Biol. 13(4):285-292.

Sugiyama T, Price JS, Lanyon LE. 2010 Functional adaptation to mechanical loading in both cortical and cancellous bone is controlled locally and is confined to the loaded bones. Bone. 46(2); 314-321.

Szulc P, Duboeuf F, Schott AM. 2006 Structural determinants of hip fracture in elderly women: re-analysis of the data from the EPIDOS study. Osteoporos Int. 17(2): 231-236.

Teitelbaum SL, Ross FP. 2003 Genetic regulation of osteoclast development and function. Nat Rev Genet. 4(8): 638-649.

Thambiah S, Roplekar R, Manghat P, Fogelman I, Fraser WD, Goldsmith D, et al. 2012 Circulating sclerostin and Dickkopf-1 (DKK1) in predialysis chronic kidney disease (CKD): relationship with bone density and arterial stiffness. Calcif Tissue Int. 90(6): 473-180.

Thorson S, Prasad T, Sheu Y, Danielson ME, Arasu A, Cummings SR, et al. 2013 Sclerostin and bone strength in women in their 10th decade of life. J Bone Miner Res. 28(9): 2008-2016.

Tian J, Xu X, Shen L, Yang YP, et al. 2015 Association of serum Dkk-1 levels with β-catenin in patients with postmenopausal osteoporosis. J Huazhong Univ Sci Technol. 35(2): 212-218.

Tolonen S, Sievanen H, Mikkila V, et al. 2015 Adolescence physical activity is associated with higher tibial pQCT bone values in adulthood after 28-years of follow-up - the cardiovascular risk in young Finns study. Bone. 75; 77-83.

Tucker LA, Strong JE, LeCheminant JD, Bailey BW. 2015 Effect of two jumping programs on hip bone mineral density in premenopausal women: a randomized controlled trial. Am J Health Promot. 29(3):158-64.

Turner CH, Robling AG. 2003 Designing exercise regimen to increase bone strength. Exer Sport Sci Rev. 31(1): 45-50.

Turner CH, Warden SJ, Bellido T, Plotkin L, Kumar Natarajan, Jasiuk I. 2009 Mechanobiology of the skeleton. Sci Signal. 2(68); pt3.

Turner CH. 1998 Three rules for bone adaptation to mechanical stimuli. Bone. 23(5); 399-407.

Ulrich D, Van Rietbergen B, Laib A, Ruegsegger P. 1999 The ability of three-dimensional structural indices to reflect mechanical aspects of trabecular bone. Bone. 25(1):55-60.

Umemura Y, Ishiko T, Yamauchi T, Kurono M, Mashiko S. 1997 Five jumps per day increase bone mass and breaking force in rats. J Bone Miner Res. 12(9); 1480–485.

Uusi-Rasi K, Sievanen H, Pasanen M, Oja P, Vuori I. 2002 Associations of calcium intake and physical activity with bone density and size in premenopausal and postmenopausal women: a peripheral quantitative computed tomography study. J Bone Miner Res. 17(3): 544-552.

Vasikaran S, Cooper C, Eastell R, Griesmacher A, Morris HA, Trenti T, Kanis JA. 2011 International Osteoporosis Foundation and International Federation of Clinical Chemistry and Laboratory Medicine position on bone marker standards in osteoporosis. Clin Chem Lab Med. 49(8):1271-1274.

Van der Horst A, Burgering BM. 2007 Stressing role of FoxO proteins in life span and disease. Nat Rev Mol Cell Biol. 8(6); 440-450.

Vielleux LN, Rauch F. 2010 Reproducibility of jumping mechanography in healthy children and adults. J Musculoskel Neur Interact. 10(4); 256-266.

Wan M, Yang C, Li J, Wu X, Yuan H, Ma H, et al. 2008 Parathyroid hormone signaling through low-density lipoprotein-related protein 6. Gene Devel. 22(21); 2968-2979.

Wang Q, Chen D, Cheng SM, Nicholson P, Alen M, Cheng S. 2015 Growth and aging of proximal femoral bone: A study with women spanning three generations. J Bone Miner Res. 30 (3): 528-534.

Warren M, Petit MA, Hannan PJ, Schmitz KH. 2008 Strength training effects on bone mineral content and density in premenopausal women. Med Sci Sports Exer. 40(7): 1282-1288.

Weeks BK, Beck BR. 2008 The BPAQ: a bone-specific physical activity assessment instrument. Osteoporos Int. 19:1567–77.

Wei W, Zeve D, Suh JM, et al. 2011 Biphasic and dosage-dependent regulation of osteoclastogenesis by beta-catenin. Mol Cell Biol. 31(23); 4706-4719.

Westendorf JJ, Kahler RA, Schroeder TM. 2004 Wnt signaling in osteoblasts and bone diseases. Gene. 341; 19-39.

Wilks DC, Winwood K, Gilliver SF., et al. 2009 Bone mass and geometry of the tibia and the radius of master sprinters, middle and long distance runners, race-walkers and sedentary control participants: A pQCT study. Bone. 45(1); 91-97.

Xu Jincheng, Lombardi G, Jia W, Banfi G. 2016 Effects of exercise on bone status in female subjecys, from young girls to postmenopausal women: an overview of systematic reviews and meta analyses. Sports Med. 46(8): 1165-1182.

Yao GQ, Wu JJ, Troiano N, Insogna K. 2011 Targeted overexpression of Dkk1 in osteoblasts reduces bone mass but does not impair the anabolic response to intermittent PTH treatment in mice. J Bone Miner Metab 29(2):141–148.

Yao W, Cheng Z, Pham A, Busse C, Zimmermann EA, Ritchie RO, et al. 2008 Glucocorticoid-induced bone loss in mice can be reversed by the actions of parathyroid hormone and risedronate on different pathways for bone formation and mineralization. 58(11); 3485-3497.

Yeh JK, Aloia JF, Chen MM, Tierney JM, Sprintz S. 1993 Influence of exercise on cancellous bone of the aged female rat. J Bone Miner Res. 8(9); 1117–1125.

Yong JC. 2016 Dual-energy X-ray absorptiometry: beyond bone mineral density determination. Endocrinol Metab. 31(1): 25-30.

Zhang Y, Paul EM, Sathyendra V, Davison A, Sharkey N, Bronson S et al. 2011 Enhanced osteoclastic resorption and responsiveness to mechanical load in gap junction deficient bone. PLoS One. 6(8); e23516.

Appendix A: Informed Consent

Informed Consent and Authorization and Use or Disclose Protected Health Information for Research

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

Wnt Signaling Inhibitor Characteristics According to Bone Status, Physical Activities Levels, and Muscle Function in Young and Middle-Aged Premenopausal Women

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

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

Why Have I Been Asked To Participate In This Study?

You are being asked to take part in this study because you are a premenopausal women in the age range of 20-30 years or 35-45 years.

Why Is This Study Being Done?

The purpose of this investigation is to compare blood markers of bone metabolism in young (20-30 years) and middle-aged (35-45 years) premenopausal women. This study will also evaluate the differences in these bone markers based on physical activity status (low, moderate, and high). Further, this study will evaluate the relationships of these bone markers with bone mineral density, leg strength, and jump power.

How Many People Will Take Part In The Study?

Approximately 50 female participants, 25 in each age group, will take part in this study.

What Is Involved In The Study?

If you take part in this study, 3-4 visits will be needed. The first visit consists of the following tests in order to determine your medical and health history related to bone health. The questionnaires will take approximately 30 minutes, followed by familiarization of muscle strength and leg power techniques that will take about 20 minutes.

- Informed Consent – must sign and date an informed consent form (this document) stating that you understand all procedures and your rights as a participant.
- HIPAA Form – must sign and date the HIPAA form that indicates that your health information is protected.
- Health Status Questionnaire (HSQ) - you may be excluded from the study if any answer on this questionnaire indicates you may not be eligible for this study.

- Physical Activity Readiness Questionnaire (PAR-Q) – you may be excluded from the study if any answer on this questionnaire indicates you may not be eligible for this study.
- International Physical Activity Questionnaire (IPAQ) - this questionnaire allows us to categorize the total amount of activity you do in a normal week.
- Bone Specific Physical Activity Questionnaire (BPAQ) – this questionnaire will be used to determine if any past activities may have an influence on your current bone health
- Calcium Intake Questionnaire – this questionnaire will be used to determine that adequate calcium is being ingested to promote normal and healthy bone.
- Menstrual History Questionnaire - will determine any menstrual characteristics that might impact bone health.
- You will be familiarized with the methods of the jump test and two leg press strength test.

The second visit (about 10 minutes) will require you to visit the Goddard Health Service building to have a venipuncture blood draw performed by a registered nurse or phlebotomist.

The third and possibly fourth visits will consist of the following tests to evaluate your bone density and body composition.. We will also assess your leg strength (two leg press), and lower body power. This visit will take about 1 ½ hours.

- Urine Pregnancy Test– will be completed in order to determine that you (the participant) are not pregnant and determine that you are eligible for bone scans. (5 minutes).
- Hydration Measurement- will also make sure you are adequately hydrated so that you can complete bone scans (5 minutes).
- Height and Weight – your height and weight will be measured (5 minutes).
- Series of bone scans using a type of x-ray machine called Dual Energy X-ray Absorptiometry (DXA) – will measure bone mineral density of your whole body, lower back, and the right and left hips. These tests are non-invasive and will take approximately 15 minutes to complete. You will be lying on your back on the DXA table for the scans and you will be required to remain still during the procedures. There are risks associated with DXA which will be addressed below.
- Series of bone scans using a different type of x-ray machine called peripheral Quantitative Computed Tomography (pQCT) – these scans will take approximately 20 minutes and will include 3 scans on your non-dominant (non-kicking) lower leg. These tests are non-invasive and only require that you sit still in a chair while the scanner measures your lower leg at three locations. The lengths of each limb will be measured in order to determine the correct positioning on the pQCT machine. The pQCT utilizes radiation and is for research purposes alone. There are risks associated with pQCT which will be addressed below.

- Jump Test - will be used to measure vertical jump height, time in the air, jump power and velocity. You will be asked to do a countermovement vertical jump by crouching, then jumping with non-restricted arm motion, and then landing on the jump mat. A minimum of 1-minute rest or as long as you need will be allowed between jumps. Trained spotters will be standing on either side of you to help with balance if needed. After several warm-up jumps, three trials will be conducted which takes about 10 minutes.
- Two leg press strength – you will perform the 1RM protocol for the leg press exercise. This test will be done after the jump test. First, you will perform a set of 8-10 repetitions at a light load. Following a 1 minute rest, you will then complete 3-4 repetitions with a load approximately equal to 75% of the estimated maximal strength. Then following a 2-minute rest, loads will be increased so that a maximal voluntary effort is achieved within 5 more attempts. This test only takes about 15 minutes.

How Long Will I Be In The Study?
The study will span 3-4 days and require approximately 3-4 hours total. The first visit will take approximately 50 minutes and consist of the consenting process, questionnaires, and a familiarization with the muscle testing procedures. The second visit will take approximately 10 minutes and will require a visit to the Goddard Health Center for a blood draw. The third and possibly fourth visit will take about 1 ½ hours and will consist of the bone scans (DXA and pQCT), and muscular strength and jump power tests.
There may be unanticipated circumstances under which your participation may be terminated by the investigator without regard to your consent. You may be terminated based on:

- medications impacting bone health
- the presence of metal implants
- recent injuries

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

What Are The Risks of The Study?
While in the study, you are at risk for these side effects; however, there may also be an unforeseeable risk with participation. You should discuss these with the researcher prior to providing your consent.

- Risks and side effects related to having bone scans (DXAand pQCT):
  This research study involves exposure to radiation from 4 DXA scans and 3 pQCT scans, which are types of x-ray procedures. These procedures are for research only and not needed for your medical care. The amount of additional radiation to which you will be exposed is approximately 1% of the amount of radiation to which we have exposed annually from background sources such as the Earth and Sun. In addition to any radiographic procedures that are being done as part of this research, you may also be exposed to radiation from procedures that are part of your normal care. The risk from radiation exposure increases over your lifetime as you receive additional exposure to radiation.

- Risks for lower body strength and jump power assessments:
  There is a slight possibility of mild soreness because of muscle strength and jump testing. Additionally, there is a slight risk of injury/fall due to jumping movements during jump test.

- Risks for blood draw:
  There may be temporary discomfort, pain, and bruising at the site of the blood draw. You may feel faint and have a slight risk of infection.

- Risks for being pregnant:
  You must not be and should not become pregnant while in this study. Participating in the bone scans (DXA and pQCT scans) involved in this study while pregnant may involve risks to an embryo or fetus, including birth defects which are currently unforeseeable. In order to reduce your risk of pregnancy, you or your partner should use one or more of the acceptable methods of birth control listed below, regularly and consistently while you are in this study.

Acceptable methods of birth control (continuing throughout the study) include:

- o An approved oral contraceptive (birth control pill)
- o Intra-uterine device (IUD)
- o Hormone implants
- o Contraceptive injection (Depo-Provera)
- o Barrier methods (diaphragm with spermicidal gel or condoms)
- o Transdermal contraceptives (birth control patch)
- o Vaginal contraception ring (birth control ring)
- o Sterilization (tubal ligation, hysterectomy or vasectomy)

If you become pregnant or suspect that you are pregnant during this study, you should immediately inform the study personnel. If you become pregnant or suspect that you are pregnant while on this study, a pregnancy test will be done. If pregnancy is confirmed, you will be withdrawn from the study.

Are There Benefits to Taking Part in The Study?
There are no direct medical benefits from participating in this study. However, information regarding your bone scans, bone markers can further provide insight to the clinical and health professionals in exploring the ways to prevent osteoporosis.

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

What About Confidentiality?
Efforts will be made to keep your personal information confidential. You will not be identifiable by name or description in any reports or publications about this study. We cannot guarantee absolute confidentiality. Your personal information may be disclosed if

required by law. You will be asked to sign a separate authorization form for use or sharing of your protected health information.

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

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

Will I Be Paid For Participating in This Study?
You will be reimbursed $10 for your time and participation in this study. If you are selected to do the fourth visit, you will be compensated an additional $5 for a total of $15 for your participation.

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

What Are My Rights As a Participant?
Taking part in this study is voluntary. You may choose not to participate. Refusal to participate will involve no penalty or loss of benefits to which you are otherwise entitled.

If you agree to participate and then decide against it, you can withdraw for any reason and leave the study at any time. However, please be sure to discuss leaving the study with the principal investigator.

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

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

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

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

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

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

Signature:

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

I agree to participate in this study:

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

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

Title of Research Project: **Wnt Signaling Inhibitor Characteristics According to Bone Status, Physical Activities Levels, and Muscle Function in Young and Middle-Aged Premenopausal Women**

Leader of Research Team: **Debra Bemben Ph.D**

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

Phone Number: **405-352-2709**

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

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

Purposes for Using or Sharing PHI. If you give permission, the researchers may use your PHI to investigate the Wnt signaling inhibitor charactersitics according to bone status, physical activities levels, and muscle function in young (20-30 years) and middle-aged (35-45 years) premenopausal women.

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

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

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

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

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

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

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

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

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

Privacy Official University of Oklahoma Health Sciences Center PO Box 26901 Oklahoma City, OK 73190	or	Privacy Board University of Oklahoma Health Sciences Center PO Box 26901 Oklahoma City, OK 73190
If you have questions, call: (405) 271-2511	or	(405) 271-2045.

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

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

Patient/Participant Name (Print): _____

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

Or

_____ _____
Signature of Legal Representative** Date

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

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

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

Appendix B: Questionnaires

Health Screening, Menstural History, Calcium Intake, International Physical Activity Questionnaire, and Bone Specific Physical Activity Questionnaire and Menstrual History

Bone Density Research Laboratory

OU Department of Health and Exercise Science

Health Status Questionnaire

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

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

Part 1. Information about the individual

1._____

Date

2. _____

Legal name Nickname

3._____

Mailing address

_____

Home phone Business/cell phone

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

5. Year of birth: _____ Age _____

6. Number of hours worked per week:

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

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

Sitting at desk Lifting or carrying loads Standing Walking Driving

Part 2. Medical history

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

Father Mother Brother Sister Grandparent

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

Year Year

IRB NUMBER: 6945
IRB APPROVAL DATE: 07/08/2016

9. Circle operations you have had:

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

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

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

11. Circle all medicine taken in last 6 months:

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

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

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

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

a. Cough up blood (MC)	d. Leg pain (MC)	g. Swollen joints (MC)
1 2 3 4 5	1 2 3 4 5	1 2 3 4 5
b. Abdominal pain (MC)	e. Arm or shoulder pain (MC)	h. Feel faint (MC)
1 2 3 4 5	1 2 3 4 5	1 2 3 4 5
c. Low back pain (SLA)	f. Chest pain (RF) (MC)	i. Dizziness (MC)
1 2 3 4 5	1 2 3 4 5	1 2 3 4 5
j. Breathless with slight exertion (MC)		
1 2 3 4 5		

Part 3. Health-related behavior

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

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

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

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

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

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

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

1. Yes 2. No

Subject ID:_____Date:_____

Bone Density Research Laboratory
Department of Health and Exercise Science
University of Oklahoma

MENSTRUAL HISTORY QUESTIONNAIRE

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

Are you pregnant (circle your response)

YES- Do not complete the rest of this form
NO- Continue to section A.

SECTION A: CURRENT MENSTRUAL STATUS

1. Approximately how many menstrual periods have you had during the past 12 months?
(please circle what months you have had a period. This means from this time last year to the present month)

Jan Feb Mar Apr May Jun Jul Aug Sep Oct Nov Dec

2. What is the usual length of your menstrual cycle (first day of your period to the next onset of your period)?

_____days. Today is day _____ of your present menstrual cycle.

3. What was the date of the onset of your last period?

4. When do you expect you next period?

5. What is the average length (number of days) of your menstrual flow? _____ days

How many of these days do you consider "heavy"?_____days

6. Do you experience cramps during menstruation (dysmenorrheal)? If yes, how many days does this last?

7. Do you experience symptoms of premenstrual syndrome (i.e., weight gain, increased eating, depression, headaches, anxiety, breast tenderness)? If yes, please list the symptoms.

8. Do you take oral contraceptives or any other medication that includes estrogen and/or progesterone?

   If yes, how long have you been taking this medication?_____

   What is the brand name and dosage of this mediation?_____

Has this medication affected your menstrual cycle (regularity, length and amount of flow)? If yes, indicate changes.

9. Have you taken oral contraceptives in the past? If no, skip to SECTION B.

   If yes, what was the brand name and dosage?_____

   When did you start taking the pill; for how long; and when did you stop taking it?

10. If you answered yes to 9 or 10, did you experience a weight gain and/or a change in appetite as a result of oral contraceptive use? If so, please indicate amount of weight gained. _____lbs

SECTION B: PAST MENSTRUAL HISTORY

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

2. Were your periods regular (occurring monthly) during the first two years after menstruation began? If not, at what age did your period become regular?

3. Has there been any time in the past where your periods were irregular or absent? If no, skip to question 4. If yes, did these periods coincide with unusual bouts of training, or with a period of stress?

4. If you have had an irregular period due to training please describe (i.e., you have a period in the offseason but only irregular menstruation during preseason and season)?

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

   Have you ever been diagnosed as having a shortened luteal phase (the time in between periods)?

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

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

CALCIUM INTAKE ESTIMATION

NAME:_____ TODAY'S DATE:_____

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

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

PLEASE TURN OVER

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

Please list below any dietary supplements (single and multi-vitamins, calcium, herbal etc.) you take daily/weekly, including the brand name, amount (mg) per dose and total number of doses per day (or per week if not taken daily).

1. _____

2. _____

3. _____

4. _____

5. _____

Bone-Specific Physical Activity Questionnaire (BPAQ)

SUBJECT ID:	DATE:

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

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

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

IRB NUMBER: 6945
IRB APPROVAL DATE: 07/08/2016

Bone-Specific Physical Activity Questionnaire (BPAQ)

SUBJECT ID:	DATE:

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

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

Activity: _____ Frequency (per week): _____

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

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE (October 2002)

LONG LAST 7 DAYS SELF-ADMINISTERED FORMAT

FOR USE WITH YOUNG AND MIDDLE-AGED ADULTS (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health–related physical activity.

Background on IPAQ

The development of an international measure for physical activity commenced in Geneva in 1998 and was followed by extensive reliability and validity testing undertaken across 12 countries (14 sites) during 2000. The final results suggest that these measures have acceptable measurement properties for use in many settings and in different languages, and are suitable for national population-based prevalence studies of participation in physical activity.

Using IPAQ

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

Translation from English and Cultural Adaptation

Translation from English is encouraged to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at www.ipaq.ki.se. If a new translation is undertaken we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

Further Developments of IPAQ

International collaboration on IPAQ is on-going and an ***International Physical Activity Prevalence Study*** is in progress. For further information see the IPAQ website.

More Information

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at www.ipaq.ki.se and Booth, M.L. (2000). *Assessment of Physical Activity: An International Perspective.* Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** and **moderate** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal.

PART 1: JOB-RELATED PHYSICAL ACTIVITY

The first section is about your work. This includes paid jobs, farming, volunteer work, course work, and any other unpaid work that you did outside your home. Do not include unpaid work you might do around your home, like housework, yard work, general maintenance, and caring for your family. These are asked in Part 3.

1. Do you currently have a job or do any unpaid work outside your home?

   ☐ Yes

   ☐ No → ***Skip to PART 2: TRANSPORTATION***

The next questions are about all the physical activity you did in the **last 7 days** as part of your paid or unpaid work. This does not include traveling to and from work.

2. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, heavy construction, or climbing up stairs **as part of your work**? Think about only those physical activities that you did for at least 10 minutes at a time.

   _____ **days per week**

   ☐ No vigorous job-related physical activity → ***Skip to question 4***

3. How much time did you usually spend on one of those days doing **vigorous** physical activities as part of your work?

   _____ **hours per day**
   _____ **minutes per day**

4. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads **as part of your work**? Please do not include walking.

   _____ **days per week**

   ☐ No moderate job-related physical activity → ***Skip to question 6***

5. How much time did you usually spend on one of those days doing **moderate** physical activities as part of your work?

_____ **hours per day**
_____ **minutes per day**

6. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **as part of your work**? Please do not count any walking you did to travel to or from work.

_____ **days per week**

☐ No job-related walking → ***Skip to PART 2: TRANSPORTATION***

7. How much time did you usually spend on one of those days **walking** as part of your work?

_____ **hours per day**
_____ **minutes per day**

PART 2: TRANSPORTATION PHYSICAL ACTIVITY

These questions are about how you traveled from place to place, including to places like work, stores, movies, and so on.

8. During the **last 7 days**, on how many days did you **travel in a motor vehicle** like a train, bus, car, or tram?

_____ **days per week**

☐ No traveling in a motor vehicle → ***Skip to question 10***

9. How much time did you usually spend on one of those days **traveling** in a train, bus, car, tram, or other kind of motor vehicle?

_____ **hours per day**
_____ **minutes per day**

Now think only about the **bicycling** and **walking** you might have done to travel to and from work, to do errands, or to go from place to place.

10. During the **last 7 days**, on how many days did you **bicycle** for at least 10 minutes at a time to go **from place to place**?

_____ **days per week**

☐ No bicycling from place to place → ***Skip to question 12***

11. How much time did you usually spend on one of those days to **bicycle** from place to place?

_____ **hours per day**
_____ **minutes per day**

12. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time to go **from place to place?**

_____ **days per week**

☐ No walking from place to place → ***Skip to PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY***

13. How much time did you usually spend on one of those days **walking** from place to place?

_____ **hours per day**
_____ **minutes per day**

PART 3: HOUSEWORK, HOUSE MAINTENANCE, AND CARING FOR FAMILY

This section is about some of the physical activities you might have done in the **last 7 days** in and around your home, like housework, gardening, yard work, general maintenance work, and caring for your family.

14. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, chopping wood, shoveling snow, or digging **in the garden or yard?**

_____ **days per week**

☐ No vigorous activity in garden or yard → ***Skip to question 16***

15. How much time did you usually spend on one of those days doing **vigorous** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

16. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, sweeping, washing windows, and raking **in the garden or yard?**

_____ **days per week**

☐ No moderate activity in garden or yard → ***Skip to question 18***

17. How much time did you usually spend on one of those days doing **moderate** physical activities in the garden or yard?

_____ **hours per day**
_____ **minutes per day**

18. Once again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** activities like carrying light loads, washing windows, scrubbing floors and sweeping **inside your home**?

_____ **days per week**

☐ No moderate activity inside home → ***Skip to PART 4: RECREATION, SPORT AND LEISURE-TIME PHYSICAL ACTIVITY***

19. How much time did you usually spend on one of those days doing **moderate** physical activities inside your home?

_____ **hours per day**
_____ **minutes per day**

PART 4: RECREATION, SPORT, AND LEISURE-TIME PHYSICAL ACTIVITY

This section is about all the physical activities that you did in the **last 7 days** solely for recreation, sport, exercise or leisure. Please do not include any activities you have already mentioned.

20. Not counting any walking you have already mentioned, during the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time **in your leisure time**?

_____ **days per week**

☐ No walking in leisure time → ***Skip to question 22***

21. How much time did you usually spend on one of those days **walking** in your leisure time?

_____ **hours per day**
_____ **minutes per day**

22. Think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **vigorous** physical activities like aerobics, running, fast bicycling, or fast swimming **in your leisure time**?

_____ **days per week**

☐ No vigorous activity in leisure time → ***Skip to question 24***

23. How much time did you usually spend on one of those days doing **vigorous** physical activities in your leisure time?

_____ **hours per day**
_____ **minutes per day**

24. Again, think about only those physical activities that you did for at least 10 minutes at a time. During the **last 7 days**, on how many days did you do **moderate** physical activities like bicycling at a regular pace, swimming at a regular pace, and doubles tennis **in your leisure time?**

_____ **days per week**

☐ No moderate activity in leisure time → ***Skip to PART 5: TIME SPENT SITTING***

25. How much time did you usually spend on one of those days doing **moderate** physical activities in your leisure time?
_____ **hours per day**
_____ **minutes per day**

PART 5: TIME SPENT SITTING

The last questions are about the time you spend sitting while at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television. Do not include any time spent sitting in a motor vehicle that you have already told me about.

26. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekday**?

_____ **hours per day**
_____ **minutes per day**

27. During the **last 7 days**, how much time did you usually spend **sitting** on a **weekend day**?

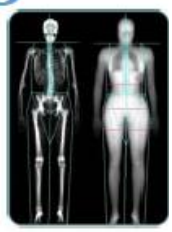
_____ **hours per day**
_____ **minutes per day**

This is the end of the questionnaire, thank you for participating.

Appendix C: Recruitment Materials

Flyer, Screening Checklist, Message Board Announcement, Email Script and Verbal Recruitment

FEMALE PARTICIPANTS NEEDED



Wnt Signaling Inhibitor Characteristics According to Bone Status, Physical Activity Levels, and Muscle Function in Young and Middle-Aged Premenopausal Women

To participate

- Women in the age range of 20-30 years or 35- 45 years old, with normal menstrual cycle
- Weight < 350 lbs and height < 6 feet and 4 inches
- Not pregnant and currently not smoking or history of smoking for past 6 months.
- Not taking medication that can affect bone density or metabolism, such as corticosteroids, glucocorticoids, thiazide diuretics, bisphosphonates, and calcitonin
- Women without artificial knee/hip joints, or other metal implants in the spine or hips, recent surgery, fracture and open wounds
- Women without physical disabilities that prevent them from performing weight lifting exercises.

Required Testing (3-4 visits)

- Informed consent, HIPAA and health related questionnaire, and familiarization with jump test and two leg press strength test (Visit 1)
- Blood sampling by venipuncture to assess serum sclerostin, DKK-1 and FSH. (Visit 2)
- Bone scans with DXA and pQCT: low-dose radiation will be used to assess bone density and Jump test and two leg press strength assessment (Visit 3 or possibly Visit 4)

There are possible risks involved with participation, including temporary soreness from blood sampling and risk associated with radiation exposure, and soreness from muscle strength and jump test.

Research participants will be compensated upon the completion of the study

If you are eligible and interested, please contact:

Pragya Sharma

Department of Health and Exercise Science
pragya@ou.edu 405-385 1160
(PI: Debra Bemben, Ph.D; dbemben@ou.edu)

The University of Oklahoma is an equal opportunity institution.

Screening Checklist

Wnt Signaling Inhibitor Characteristics According to Bone Status, Physical Activity Levels, and Muscle Function in Young and Middle-Aged Premenopausal Women.

Name: _____ Date: _____

Does participant meet the inclusion criteria for the study?

	Yes	No
Age 20-30 or 35-45 years	____	____
Normal Menstrual Cycles (10-14 cycles in 12 months)	____	____
Weight less than 350 lbs	____	____
Height less than 6 feet and 4 inches	____	____

Does participant have any exclusion criteria?

Women who are pregnant	____	____
Current smokers or those who had smoked regularly within the past 6 months	____	____
Women taking medication that can affect bone density or metabolism, such as corticosteroids, glucocorticoids, thiazide diuretics, bisphosphonates, and calcitonin	____	____
Women with artificial knee/hip joints, or other metal implants in the spine or hips	____	____
Women with recent surgery, fracture, and open wounds	____	____
Women with physical disabilities that prevent them from performing weight lifting exercises	____	____
Qualified for study	____	____

PI Signature _____ Date _____

Mass e-mail script

We are looking for healthy women between the ages of 20- 30 or 35-45 years old. Potential participants must have normal menstrual cycles (10-14 cycles in 12 months), be non-smokers, weigh less than 350 lbs and be less than 6 feet and 4 inches tall. Additionally, potential participants should not be pregnant, and not taking medications that can affect bone density. Participants will also be excluded if they have artificial knee/hip joints or other metal implants in the spine or hips. Women with recent surgeries, fractures, or open wounds and those physical disabilities that prevent them from performing weight lifting exercises will also be excluded from this study.

This study requires 3-4 visits for a total time commitment of about 3-4 hours (visit 1 = 50 minutes; visit 2 = 10 minutes; visit 3-4 = 90 minutes). One venipuncture blood draw (about 7 ml) for each participant will be performed by a registered nurse or phlebotomist at the OU Goddard Health Center in the morning after an overnight fast to measure serum sclerostin, serum DKK-1, and serum FSH concentrations. This study requires exposure to a small amount of radiation by 2 different machines DXA and pQCT and you will have a total of 7 bone scans to assess your bone health. Participants will perform the jump test and two leg press strength test protocols to assess the neuromuscular performance.

There are possible risks involved with participation, including risks associated with radiation exposure, strength and power testing, and the blood draw. Tthere is a slight risk of injury/fall due to jumping movements during jump test. There is a slight possibility of mild soreness because of the muscle strength and power testing. There may be temporary discomfort, pain, and bruising at the site of the blood draw. Participants may feel faint and have a slight risk of infection from the blood draw. Information regarding your results will be provided at the end of the study upon your request. You will be reimbursed $10 for your time and participation in this study. If you are selected to do the fourth visit, you will be compensated an additional $5 for a total of $15 for your participation.

If you are interested and for more information please contact Pragya Sharma e-mail: pragya@ou.edu.

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

IRB NUMBER: 6945
IRB APPROVAL DATE: 09/10/2015

Telephone script

1. My name is Pragya Sharma-Ghimire and I am a graduate student from the University of Oklahoma.
2. Is _____available?
    - (If yes) I was informed that you may be interested in participating in a bone study. Is this correct? (if yes go to question 3).
    - (If no) Is this the correct number to reach ___?
    - (If no) I apologize, please have a great day.
    - (If yes) Do you know a better time for me to call back to reach ____?
        - (if no) Thank you for your time.
        - (If yes) I was calling to give you more information about the study and the requirements of being a participant.
3. Do you have time to talk right now?
    - (If no) What is a better time for me to contact you?
    - (If yes) Let me tell you a little about the study. The purpose of this investigation is to compare the markers of bone metabolism such as sclerostin and Dickkopf-related protein 1 (DKK-1) in young (20-30 years) and middle-aged (35-45 years) premenopausal women. This study includes a series of scans with two machines: DXA and pQCT. You will be exposed to small amount of radiation. I can give you the exact details if you wish. I will conduct jump test and two leg press strength to assess your muscle power and strength. In addition, there is one venipuncture blood draw required in OU Goddard to measure your serum sclerostin and DKK-1. However, before we proceed, I have to tell you that I will be collecting your answers, if for some reason you chose to not participate or you do not qualify to participate your name, phone number and the reason you will not be participating will be recorded. This is just for assurance that we are treating all possible participants fairly, thus, your information will be kept private and only used for this research study. If you do not wish to answer questions over the phone I would be more than happy to meet with you in person at the bone density laboratory on the Norman campus, or I can send you more information via e-mail. Please remember that your participation should be voluntary. Would you like to continue?
        - (If no) I thank you for your time.
        - (if yes)
    - First, I am going to ask you a series of questions to determine your eligibility.
4. How old are you? Are you between 20-30 or 35-45 years old?
        - (If no) Thank you for your time
        - (if yes) Let me ask some questions about the inclusion criteria.
5. Do you have a normal menstrual cycle (10-14 cycles in 12 months)?

- (If no) Thank you for your time
- (if yes) Let me ask some questions about the inclusion criteria.

6. Is your weight is less than 350 lbs and height less than 6 feet and 4 inches?
   - (If no) Thank you for your time
   - (if yes) Let me ask some questions about the exclusion criteria.
7. Are you pregnant or planning to be pregnant?
8. Are you a current smoker? Have you smoked regularly within the past 6 months?
9. Are you taking medication that can affect bone density or metabolisms, such as corticosteroids, glucocorticoids, thiazide diuretics, bisphosphonates, and calcitonin?
10. Do you have artificial knee/hip joints, or other metal implants in the spine or hips?
11. Do you have recent surgery, fracture, and open wounds?
12. Do you have physical disabilities that prevent them from performing weight lifting exercises?

Thank you for your responses, at this time you (do/do not) qualify for this study

they qualify:

Do you have time for me to tell you about what you will be expected to do for this study? (if no) When is a better time for me to call back? Thank you for your time.

(if yes proceed to describing the study)

Testing includes 3 to 4 visits to the Bone Density Lab at the University of Oklahoma. During the first visit, the participants will sign and date the informed consent and HIPAA forms and complete health-related questionnaires. Trained personnel will instruct participants on correct techniques for the two leg press, and the jump test. After the instruction, participants will practice at light intensities to get accustomed to the movements. During the second visit, one venipuncture blood draws (about 7 ml) for each participant will be performed by a registered nurse or phlebotomist at the OU Goddard Health Center in the morning after overnight fasting to measure serum sclerostin, serum DKK-1, and serum FSH concentrations. During the third or possibly fourth visit, participants will have their urine pregnancy test, hydration measurement, and anthropometric measurement. Following this, you will have a total of 7 bone scans measured by 2 different machines to assess your bone health. The bone density of your total body, lumbar spine, and both hips will be measured by dual energy x-ray absorptiometry (DXA).The bone density of one of your lower legs will be measured at 3 places using peripheral Quantitative Computed Tomography (pQCT), another type of bone scanner. Finally, you will perform jump test and two leg press strength test protocols to assess the neuromuscular performance. All testing procedures will be performed by qualified personnel. Visit 1 will take about 50 minutes to complete, visit 2 will take about 10 minutes to complete, and visits 3-4 will take about 1.5 hours each to complete. There are possible risks involved with participation, including risks associated with radiation exposure, strength and power testing, and blood draw. Additionally, there is a

slight risk of injury/fall due to jumping movements during jump test. There is also a slight possibility of mild soreness because of the muscle strength and power testing. There may be temporary discomfort, pain, and bruising at the site of the blood draw. You may feeling faint and have a slight risk of infection. Information regarding your results will be provided at the end of the study upon your request.

Facebook.com

We are looking for healthy women between the ages of 20-30 or 35-45 years old. Potential participants must have normal menstrual cycles (10-14 cycles in 12 months), be non-smokers, weigh less than 350 lbs and be less than 6 feet and 4 inches tall. Additionally, potential participants should not be pregnant, and not be taking medications that can affect bone density. Participants will also be excluded if they have artificial knee/hip joints or other metal implants in the spine or hips. Women with recent surgeries, fractures, or open wounds, and those with physical disabilities that prevent them from performing weight lifting exercises will also be excluded from this study.

This study requires 3-4 visits for a total time commitment of about 3-4 hours (visit 1 = 50 minutes; visit 2 = 10 minutes; visit 3-4 = 90 minutes). One venipuncture blood draw (about 7 ml) for each participant will be performed by a registered nurse or phlebotomist at the OU Goddard Health Center in the morning after an overnight fast to measure serum sclerostin, serum DKK-1, and serum FSH concentrations. This study requires exposure to a small amount of radiation by 2 different machines DXA and pQCT and you will have a total of 7 bone scans to assess your bone health. Participants will perform the jump test and two leg press strength test protocols to assess the neuromuscular performance.

There are possible risks involved with participation, including risks associated with radiation exposure, strength and power testing, and the blood draw. There is a slight risk of injury/fall due to the jumping movements during jump test. There is a slight possibility of mild soreness because of the muscle strength and power testing. There may be temporary discomfort, pain, and bruising at the site of the blood draw. Participants may feel faint and have a slight risk of infection from the blood draw. Information regarding your results will be provided at the end of the study upon your request. You will be reimbursed $10 for your time and participation in this study. If you are selected to do the fourth visit, you will be compensated an additional $5 for a total of $15 for your participation. If you are interested in this study and for more information, please contact Pragya Sharma via email pragya@ou.edu.

The University of Oklahoma is an equal opportunity institution

IRB NUMBER: 6945
IRB APPROVAL DATE: 09/10/2016

Appendix D: Precision

Precision for Body Composition and Bone Density Variables

Variable	%CV
%BF	2.07%
Fatmass	2.01%
BFLBM	1.94%
Fatfree Mass	1.74%
Total Body BMD	0.71%
Total BMC	0.55%
Spine BMD	1.45%
Dual Femur BMD	0.67%
Spine BMC	1.52%
Right Neck	0.91%
Left Neck	1.01%
Right Trochanter	0.77%
Left Trochanter	1.04%
Total Left	0.68%
Total Right	0.61%
Right SI	4.63%
Right Buckling Ratio	2.9%
Right SM	2.35%
Right CSMI	2.42%
Left SI	5.2%
Left Buckling Ratio	17%
Left SM	2.33%
Left CSMI	2.74%
4% Total vBMC	1.19%
4% Total vBMD	1.01%
4% Total Area	1.86%
4% Trab Area	1.93%
4% Peri C	1.39%
4% BSI	1.17%

4% Total vBMC	1.19%
4% Total vBMD	1.01%
4% Total Area	1.86%
4% Trab Area	1.93%
4% Peri C	1.39%
4% BSI	1.17%
4% Trab BSI	1.47%
38% Total vBMC	0.33%
38% Total vBMD	0.21%
38% Total Area	0.38%
38% Peri C	0.19%
38% Endo C	0.35%
38% Cort vBMC	0.36%
38% Cort vBMD	0.20%
38% Cort Area	0.47%
38% SSI	0.88%
38% IPolar	0.71%
66% Total vBMC	0.24%
66% Total vBMD	0.67%
66% Total Area	0.67%
66% Peri C	0.33%
66% Endo C	0.74%
66% Cort vBMC	0.34%
66% Cort vBMD	0.25%
66% Cort Area	0.38%
66% SSI	0.93%
66% IPolar	1.11%
66% MCSA	1.47%

Appendix E: Correlation

Correlation between sclerostin/DKK-1 with hip structural analyses and pQCT variables

Variables	r	Variables	r
Serum sclerostin and		Serum DKK-1 and	
Right Strength Index	0.06	Right Strength Index	0.13
Right Buckling Ratio	-0.75	Right Buckling Ratio	0.08
Right Sectional Modulus	0.07	Right Sectional Modulus	0.17
Right CSMI	-0.31	Right CSMI	0.19
Left Strength Index	0.07	Left Strength Index	0.05
Left Buckling Ratio	0.07	Left Buckling Ratio	-0.15
Left Sectional Modulus	0.04	Left Sectional Modulus	0.15
Left CSMI	0.03	Left CSMI	0.12

Variables	r	Variables	r
Serum sclerostin and		Serum DKK-1 and	
Total vBMC	0.03	Total BMC	0.15
Total vBMD	0.07	Total vBMD	0.09
Trabecular BMC	-0.07	Trabecular BMC	0.02
Trabecular vBMD	-0.06	Trabecular vBMD	-0.03
Total Area	-0.02	Total Area	0.12
Trabecular Area	-0.05	Trabecular Area	0.07
Periosteal Circumference	-0.00	Periosteal Circumference	0.13
Bone Strength Index	0.06	Bone Strength Index	0.13
Trabecular Bone Strength Index	-0.08	Trabecular Bone Strength Index	-0.01

Appendix F: Package Inserts

Package Inserts for Sclerostin, DKK-1 and FSH assays

Day 2

REF
Catalog Number

Manufacturer

Consult Instructions for Use

CONT
Contents / contains

Contains sufficient for <n> tests

Biological risks

Temperature Limitation

REF TE1023HS – TECOmedical Sclerostin HS Enzyme Immunoassay Kit

Manufactured by
Quidel Corporation | 10165 McKellar Court
San Diego, CA 92121 USA | quidel.com
For
TECOmedical Group
QUIDEL
1481A(2013/04)

TECOmedical Group Human Sclerostin HS EIA Kit

always your partner

An immunocapture enzyme assay for the determination of sclerostin in human serum and plasma

For Research Use Only.
Not for Use in Diagnostic Procedures.

8°C 46°F
2°C 36°F

REF TE1023HS

1. Robling A.G., Bellido T., Turner C.H., 2006. "Mechanical stimulation in vivo reduces osteocyte expression of sclerostin." *J Musculoskelet Neuronal Interact.* 6(4):354

2. Robling A.G., Niziolek P.J., Baldridge L.A., Condon K.W., Allen M.R., Alam I., Mantila S.M., Gluhak-Heinrich J., Bellido T.M., Harris S.E., Turner C.H. 2008. "Mechanical stimulation of bone in vivo reduces osteocyte expression of SOST/Sclerostin." *J Biol Chem.* 283(9):5866-5875.

3. Hamersma H., Gardner J., Beighton P. 2003. "The natural history of sclerostosis." *Clin Genet.* 63(3)L192-197.

4. van Bezooijen R.L., Bronckers A.L., Gortzak R.A., Hogendoorn P.C.W., van der Wee-Pals L., Balemans W., Oostenbroek H.J., Van Hul W., Hamersma H., Dikkers F.G., Hamdy N.A.T., Papapoulos S.E., Lowik C.W.G.M. 2009. "Sclerostin in mineralized matrices and van Buchem disease." *J Dent Res.* 88(6):569-574.

5. Li X., Ominsky M.S., Warmington K.S., Morony S., Gong J., Cao J., Gao Y., Shalhoub V., Tipton B., Haldankar R., Chen Q., Winters A., Boone T., Geng Z., Niu Q-T., Ke H.Z., Kostenuik P.J., Simonet W.S., Lacey D.L., Paszty C. 2009. "Sclerostin antibody treatment increases bone formation, bone mass and bone strength in a rat model of postmenopausal osteoporosis." *J Bone Miner Res.* 24(4):578-588.

6. Tian X., Setterberg R.B., Li X., Paszty C., Ke H.Z., Jee W.S.S. 2010. "Treatment with a sclerostin antibody increases cancellous bone formation and bone mass regardless of marrow composition in adult female rats." *Bone* 47(3):529-533.

7. Agholme F., Li X., Isaksson H., Ke H.Z., Aspenberg P. 2010. "Sclerostin antibody treatment enhances metaphyseal bone healing in rats." *J Bone Miner Res.* 25(11):2412-2418.

8. Padhi D., Jing G., Stouch B., Fang L., Posvar E. 2011. "Single-dose, placebo-controlled, randomized study of AMG 785, a sclerostin monoclonal antibody." *J Bone Miner Res.* 26(1):19-26.

9. Tian X., Jee W.S.S., Li X., Pascty C., Ke H.Z. 2011. "Sclerostin antibody increases bone mass by stimulating bone formation and inhibiting bone resorption in a hindlimb-immobilization rat model." *Bone* 48(2):197-201.

10. Ominsky M.S., Li C., Li X., Tan H.L., Lee E., Barrero M., Asuncion F.J., Dwyer D., Han C-Y., Vlasseros F., Samadfam R., Jolette J., Smith S.Y., Stolina M., Lacey D.L., Simonet W.S., Paszty C., Li G., Ke H.Z. 2011. "Inhibition of sclerostin by monoclonal antibody enhances bone healing and improves bone density and strength of nonfractured bones." *J Bone Miner Res.* 26(5):1012-1021.

SUMMARY AND EXPLANATION

The Human Sclerostin High Sensitivity (HS) Enzyme Immunoassay is a 96-well, direct-capture immunoassay for the measurement of Sclerostin in human serum, plasma and cell culture (osteocytes and chondrocytes). Sclerostin is the protein product of the SOST gene, which is located at 17q12-21 and highly conserved across vertebrate species. The highest expression of sclerostin throughout the adult skeleton has been observed in hypertrophic chondrocytes and osteocytes. Sclerostin blocks canonical Wnt signaling by binding to the Wnt coreceptors LRP5/6, inhibiting bone formation by regulating osteoblast function and promoting osteoblast apoptosis.[1-3] Sclerostin also antagonizes bone morphogenetic protein (BMP) action (e.g. osteoblast differentiation), but does not inhibit direct BMP-induced responses.[4-7] Sclerostin expression is down-regulated by Parathyroid hormone (PTH), as well as, by the mechanical stimulation of bone.[8-12] Reduced expression of sclerostin can result in van Buchem disease, while a complete absence results in Sclerosteosis. Patients affected by Sclerosteosis show progressive hyperostosis and sclerosis of the skull, mandible and all long bones. Bone mineral density (BMD), bone volume, bone formation rate, and bone strength are significantly increased, while overall skeletal morphology appears to be normal.[13-14] A predominance of sclerostin causes reduced bone quality (Osteoporosis pseudoglioma (OPPG) syndrome). Down-regulation of sclerostin might be used as a treatment for diseases such as osteoporosis, promote osseointegration of implants, prevent prosthetic bone loss, or treat non-union in fractures.[15-20] Local enhancement of sclerostin expression might be used to prevent cancer metastasis and minimize further expansion of ectopic bone formation.[21]

PRINCIPLE OF THE PROCEDURE

The Human Sclerostin HS Enzyme Immunoassay for the quantitation of Sclerostin in human plasma and serum is a two-step procedure utilizing (1) a microassay plate coated with streptavidin and a biotinylated goat polyclonal antibody that binds specifically to human Sclerostin, (2) a HRP-conjugated monoclonal anti-human Sclerostin antibody, and (3) a chromogenic substrate.

Prior to Step 1, The microassay plate is pre-washed for 2 minutes, the wash buffer aspirated and the remaining liquid removed by tapping on absorbent paper.

In Step 1, Standards, Controls, and test specimens are added to microassay wells pre-coated with streptavidin. Biotin-conjugated primary polyclonal anti-human Sclerostin antibody and horseradish peroxidase (HRP)-conjugated secondary monoclonal anti-human Sclerostin antibody is added to each test well. Sclerostin present in the Standards, Controls or specimens are captured in the microassay wells through binding of the biotinylated primary antibody to the streptavidin immobilized on the plate and simultaneously detected by the HRP-conjugated secondary antibody. After a 4 hour incubation, a wash cycle removes unbound material.

Linearity

Linearity was performed by diluting samples with specimen diluent and comparing observed values with expected values. Typical results are provided below.

Sample	Dilution Factor	Observed Sclerostin (ng/mL)[1]	Expected Sclerostin (ng/mL)[1]	Recovery (%)
Serum 1	1	1.80	*	*
	2	0.88	0.90	98
	4	0.44	0.45	98
	8	0.22	0.23	100
Serum 2	1	2.34	*	*
	2	1.11	1.17	95
	4	0.56	0.59	96
	8	0.30	0.29	103
Serum 3	1	1.09	*	*
	2	0.54	0.55	99
	4	0.29	0.27	106
	8	0.15	0.14	108

[1]Dilution factor not included.
*Intentionally left blank.

Spike Recovery

Spike Recovery was performed by spiking samples with a known quantity of purified Sclerostin and comparing observed values with expected values.

Sample	Sclerostin (ng/mL)	Spike (ng/mL)	Result (ng/mL)	Recovery (%)
Serum 1	0.59	1.12	1.63	96
Serum 2	2.50	1.12	3.47	96
Serum 3	1.12	1.12	2.27	102

Species Cross-reactivity

Serum from different species were tested in the Human Sclerostin HS Assay. Results are provided below.

Sample	Sclerostin (ng/mL)
Bovine	0.00
Goat	0.00
Canine	0.13
Chicken	0.06
Guinea Pig	0.00
Rabbit	0.00
Rat	0.00
Mouse	0.00
Baboon	0.35
Female Cynomolgus Monkey	0.10
Sheep	0.00
Pig	0.00
Rhesus Monkey	0.36
African Green Monkey	0.00
Male Cynomolgus Monkey	0.59

[1]Concentrations >0.10 ng/mL = Cross-reactivity

MATERIALS REQUIRED BUT NOT PROVIDED

- Timer (60 minute range)
- Container and graduated cylinder for wash buffer dilution
- Wash bottle or other validated immunoassay washing system
- Micropipettes and disposable pipette tips
- Adjustable multichannel pipette (8 or 12 channels) or repeating micropipettes
- Reagent reservoirs for adding conjugate, substrate and stop solutions to plate (use clean, unused reservoirs for each reagent)
- Plate reader capable of A_{450} readings from 0.0 to at least 3.0 (Reference filter 590-650 nm)
- Deionized or distilled water
- Vortex mixer
- ELISA plate shaker (orbital shaker; 500 rpm)

WARNINGS AND PRECAUTIONS

1. *For Research Use Only. Not for use in diagnostic procedures.*
2. Treat specimen samples as potentially biohazardous material. Follow Universal Precautions when handling contents of this kit and any patient samples. Since no test method can offer complete assurance that infectious agents are absent, these materials should be handled at Biosafety Level 2 as recommended for any potentially infectious human serum or blood specimen in the Centers for Disease Control/National Institutes of Health manual, "Biosafety in Microbiological and ...medical Laboratories."[22,23]
3. ...aterial of animal origin used in the preparation of this kit has been obtained from animals certified as healthy, but these materials should be handled as potentially infectious.
4. Wear suitable protective clothing, gloves, and eye/face protection when handling contents of this kit.
5. Use the supplied reagents as an integral unit prior to the expiration date indicated on the package label.
6. Store assay reagents as indicated.
7. Do not use Coated Strips if pouch is punctured.
8. ProClin® 300 is used as a preservative. Incidental contact with or ingestion of buffers or reagents containing ProClin® can cause irritation to the skin, eyes, or mouth. Use good laboratory practices to reduce exposure. Seek medical attention if symptoms are experienced.
9. The Stop Solution is considered corrosive and can cause irritation. Do not ingest. Avoid contact with eyes, skin, and clothing. If contact is made, immediately rinse affected area with water. If ingested, call a physician.
10. Use of multichannel pipettes or repeat pipettors is recommended to ensure timely delivery of reagents.
11. For accurate measurement of samples, add samples and standards precisely. Pipette carefully using only calibrated equipment.

Sample	Area	ng/mL
Standard A	0.021	0
Standard B	0.072	0.05
Standard C	0.221	0.20
Standard D	0.568	0.50
Standard E	1.742	1.50
Standard F	3.108	3.00

Calculation of Actual Sclerostin Concentration in Test Specimens

The actual Sclerostin concentration present in each undiluted test specimen is determined from the Kit Standard Curve.

LIMITATIONS

The Human Sclerostin HS Enzyme Immunoassay has been used to test specimens collected as serum.

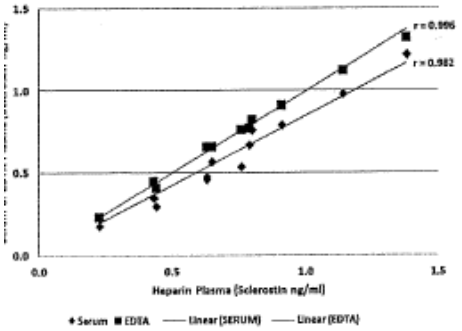
OBSERVED VALUES

Serum from normal donors were tested in the Human Sclerostin HS Enzyme Immunoassay kit. The results are presented below.

Group	n	Mean (ng/mL)	SD (ng/mL)
Premenopausal women	24	0.59	0.23
Postmenopausal women	20	0.66	0.22
Men	11	0.83	0.22

NOTE: The mean and Standard Deviation (SD) behavior of sclerostin concentrations determined for serum samples may vary between laboratories. Therefore, it is recommended that each laboratory determine the mean sclerostin concentration and standard deviation values for samples.

Figure 2: Sclerostin Values



Wash Buffer

Mix the 20X Wash Buffer Concentrate by inverting the bottle several times. If the 20X Wash Buffer Concentrate has been stored at 2-8°C, crystals may have formed. To dissolve the crystals, warm the bottle in a 37-50°C water bath until all crystals have dissolved, and follow by mixing thoroughly. Prepare the Wash Buffer by diluting the entire contents of the bottle of 20X Wash Buffer concentrate up to one liter with distilled or deionized water. Mix thoroughly. The Wash Buffer is stable for 30 days when stored in a clean container at 2-8°C. If discoloration or cloudiness occurs, discard the reagent.

Standards and Controls

Standards and Controls are supplied ready to use and do not require dilution or preparation prior to use.

SPECIMEN HANDLING AND PREPARATION

Handle and dispose of all specimens using Universal Precautions.

Specimen Collection and Storage

Plasma (Heparin and EDTA) and serum have been used as samples in the Human Sclerostin HS Assay. Collect specimens using standard venipuncture techniques. Specimens should be collected in such a way to avoid hemolysis. For serum specimens, allow the blood to clot, and separate the serum by centrifugation. Both Heparin and EDTA plasma can be used. See OBSERVED VALUES section for more information.

Stability of Samples

Samples can be stored for 3 days at room temperature, 5 days at 2-8°C, at ≤ -20°C for 24 months and at ≤ -80°C for > 24 months. Up to three thaw cycles may be performed without affecting the samples. If samples need additional freezing for further analysis, We suggest freezing multiple aliquots of the specimen to prevent exceeding the recommended number of freeze/thaw cycles.

CAUTION: Treat all specimens as potentially infectious. Use Universal Precautions. Do not use contaminated or improperly stored specimens.

Normal Specimens must not be diluted. Observed values should be above the LLOQ and not exceed the ULOQ.

Specimens with high levels of sclerostin (above the standard curve) may require dilution with Sample Diluent and retesting.

ASSAY PROCEDURE

Read entire product insert before beginning the assay.

See *Reagent Preparation* and *Warnings And Precautions*.

1. Record the microassay well positions corresponding to all test samples, Standards, and Controls, as well as the indicated lot numbers from the vial labels. Label one corner of the Microassay Plate for orientation.

Eagle Biosciences, Inc.
20A NW Blvd. Ste 112
Nashua, NH 03063
EagleBio.com
866-411-8023
617-418-1110 (fax)

BI-20413 DKK-1
ASSAY PROTOCOL AND CHECKLIST

PREPARATION OF REAGENTS:

- ☐ Bring all reagents to room temperature (18-24°C).
- ☐ Prepare reagents as instructed.
- ☐ Bring unused and prepared components to the storage temperature mentioned in the package insert.
- ☐ Take microtiter strips out of the aluminium bag and mark positions on the protocol sheet.

TEST PROCEDURE:

- ☐ Step 1) Pipette 50 µl ASYBUF (Assaybuffer) into each well.
- ☐ Step 2) Pipette 20 µl STD/SAMPLE/CTRL (standard/sample/control) into each well.
- ☐ Step 3) Add 50 µl AB (biotinylated anti DKK-1) into all wells, swirl gently.
- ☐ **Step 4) Cover tightly and incubate for 2 hours at RT (18-24°C).**
- ☐ Step 5) Aspirate and wash wells with 300 µl WASHBUF (Wash buffer) five times. Remove remaining buffer by hitting plate against paper towel.
- ☐ Step 6) Add 100 µl CONJ (Conjugate) into each well.
- ☐ **Step 7) Cover tightly and incubate for 1 hour at RT (18-24°C).**
- ☐ Step 8) Aspirate and wash wells with 300 µl WASHBUF (Wash buffer) five times. Remove remaining buffer by hitting plate against paper towel.
- ☐ Step 9) Add 100 µl SUB (Substrate) into each well.
- ☐ **Step 10) Incubate for 30 minutes at RT (18-24°C) in the dark.**
- ☐ Step 11) Add 50 µl STOP (Stop solution) into each well.
- ☐ Step 12) Read Optical Density at 450 nm with reference 630 nm, if available.

DKK-1

(EN) ENZYME IMMUNOASSAY FOR THE QUANTITATIVE DETERMINATION OF HUMAN DKK-1 IN SERUM
CAT. NO. BI-20413. 12 X 8 TESTS

(DE) ENZYM IMMUNOASSAY ZUR QUANTITATIVEN BESTIMMUNG VON HUMAN DKK-1 IN SERUM
KAT. NR. BI-20413. 12 X 8 TESTE

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

rev.no. 150126 (replacing 130823)

Biomedica Medizinprodukte GmbH & Co KG, A-1210 Wien, Divischgasse 4
Tel. +43/1/291 07 45, Fax +43/1/291 07 85, E-mail export@bmgrp.com

BIOMEDIC

) VORSICHTSMASSNAHMEN

Alle Bestandteile humanen Ursprunges wurden auf HIV-Ak und HBsAg getestet und negativ gefunden.
otzdem sollten die Reagenzien als potentiell infektiös behandelt werden.
e flüssigen Reagenzien enthalten ≤0,01% Proclin 300 als Konservierungsmittel.
rmeiden Sie Kontakt mit Augen, Haut und Schleimhaut. Proclin 300 ist in der verwendeten Konzentration nicht
isch. Allergische Reaktionen sind möglich.

Nicht mit dem Mund pipettieren.

Nicht Rauchen, Essen, Trinken oder Kosmetika während der Verwendung der Testreagenzien benutzen.

Verwenden Sie Handschuhe zur Vermeidung jedes Kontaktes zu Reagenzien.

Schwefelsäure reizt Augen und Haut. Bei Berührung gründlich mit Wasser spülen.

) LITERATUR

Siehe Kapitel 13) LITERATURE im englischen Teil des Beipacktextes.

1) INTRODUCTION

Dickkopf-1(DKK-1) is a 28.67 Da secreted protein that acts as soluble inhibitor of the WNT signaling pathway. This pathway contains lipid-modified glycoproteins that activate cell surface receptor-mediated signal transduction to regulate cell activities like: cell fate, proliferation, migration, polarity and gene expression. DKK-1 regulates developmental processes of all kinds. Thus, DKK-1 is also involved in the regulation of bone metabolism as it effects osteoblast differentiation and in regulation of tumourgenetic activity.

2) CONTENTS OF THE KIT

CONT	KIT COMPONENTS	QUANTITY
PLATE	Mouse monoclonal anti human DKK-1 antibody pre-coated microtiter strips in strip holder packed in aluminium bag with desiccant	12 x 8 tests
WASHBUF	Wash buffer concentrate 20x, natural cap	1 x 50 ml
ASYBUF	Assay buffer, red cap, ready to use	1 x 10 ml
AB	biotinylated DKK-1 antibody, green cap, ready to use	1 x 7 ml
STD	Standards 1-6 (0, 10, 20, 40, 80, 160 pmol/l), white caps, lyophilised	6 vials
CTRL	Control, yellow cap, lyophilised, exact concentration after reconstitution see label	1 vial
CONJ	Conjugate (streptavidin-HRPO), amber cap, ready to use	1 x 13 ml
SUB	Substrate (TMB solution), blue cap, ready to use	1 x 13 ml
STOP	STOP solution, white cap, ready to use	1 x 7 ml

3) ADDITIONAL MATERIAL IN THE KIT

- 2 self-adhesive plastic films
- QC protocol
- Protocol sheet
- Instruction manual for use

4) MATERIAL AND EQUIPMENT REQUIRED BUT NOT SUPPLIED

- Precision pipettes calibrated to deliver 20 µl, 50 µl, 100 µl, 200 µl and disposable tips
- ELISA reader for absorbance at 450 nm (or from 450 nm to 630 nm)
- Graph paper or software for calculation of results
- Plate washer is recommended for washing, alternative multichannel pipette or manifold dispenser
- Distilled or deionised water

5) REAGENTS AND SAMPLE PREPARATION

The assay has been validated for the use of serum samples.

All reagents and non-reconstituted STDs and CTRL are stable at 4°C (2-8°C) until expiration date stated on the label of each reagent.

Sample preparation:

Collect venous blood samples by using standardized blood collection tubes for serum. Allow samples to clot for 30 minutes at room temperature before performing serum separation by centrifugation, e.g. 20 min at 2000 x g, preferably at 4°C (2-8°C).

Measure the acquired samples immediately or aliquot samples in polypropylene tubes and store at -25°C or lower. Avoid more than three freeze-thaw cycles. Lipemic or haemolysed samples may give erroneous results. Samples should be mixed well before assaying.

If samples read higher than STD6, we recommend to dilute serum samples with STD1 and to test again.

For further information on sample stability please visit our website www.bmgrp.com/technical file or contact our customer service by e-mail export@bmgrp.com or by phone +43/ 1/ 29107-45.

Reconstitution/Handling:

STD (Standards) and CTRL (Control): Add 200 µl deionised or distilled water into each vial, reconstitute/let room temperature (18-24°C) for 15 min. Swirl gently. Take care of complete dissolving of lyophilisate. The reconstituted STDs and CTRL shall be stored at -25°C or lower until expiry date stated on the label. **Avoid more than one**

ekonstitution/Handhabung:

) (Standard) und CTRL (Kontrolle): Lösen Sie das Lyophilisat in jeweils 200 µl destilliertem Wasser bei
mmtemperatur (18-24°C) für 15 Minuten. Achten Sie auf vollständige Auflösung des Lyophilisates.Die
nstituierten STD und CTRL können bei -25°C oder niedriger bis zum Ablaufdatum gelagert werden.
meiden Sie mehr als einen Frier-Tau Zyklus.

SHBUF (Waschpuffer): Das mitgelieferte Konzentrat wird 1:20 verdünnt (zB. 50 ml WASHBUF + 950 ml
illiertes Wasser). Kristalle im Pufferkonzentrat lösen sich bei Raumtemperatur auf. Der verdünnte Puffer ist bei
(2-8°C) bis zum Ablaufdatum haltbar. Im Testsystem darf nur verdünnter WASHBUF (Waschpuffer) verwendet
den.

'ESTPRINZIP

iehe Kapitel 6) PRINCIPLE OF THE ASSAY im englischen Teil des Beipacktextes.

'ESTPROTOKOLL

Test dürfen nur Reagenzien und Proben verwendet werden, welche Raumtemperatur (18-24°C) aufweisen.
rkieren Sie die Positionen für STD/PROBE/CTRL (Standard/Probe/Kontrolle) am Protokoll Blatt.
hmen Sie die benötigten Mikrotiterstreifen aus dem Aluminium Beutel. Nicht verwendete Mikrotiterstreifen nnen mit Trockenmittel im Aluminium Beutel auf 4°C (2-8°C) bis zum angegebenen Ablaufdatum gelagert rden.
Pipettieren Sie 50 µl ASYBUF (Assaybuffer) in alle wells.
Pipettieren Sie 20 µl STD/ SAMPLE/CTRL (Standard/ Probe/Kontrolle) in Doppelbestimmung in die Mikrotiterstreifen.
Pipettieren Sie 50 µl AB (biotinylierter DKK-1 Antikörper) in alle Wells, gut mischen.
Streifen abdecken und 2 Stunden bei Raumtemperatur (18-24°C) inkubieren.
Inhalt der Wells verwerfen und 5x mit 300 µl verdünntem WASHBUF (Waschpuffer) waschen. Nach dem letzten Waschschritt Reste von Waschpuffer durch Ausklopfen auf saugfähigem Papier entfernen.
Pipettieren Sie 100 µl CONJ (Konjugat) in alle Wells.
Streifen abdecken und 1 Stunde bei Raumtemperatur (18-24°C) inkubieren.
Inhalt der Wells verwerfen und 5x mit 300 µl verdünntem WASHBUF (Waschpuffer) waschen. Nach dem letzten Waschschritt Reste von Waschpuffer durch Ausklopfen auf saugfähigem Papier entfernen.
Pipettieren Sie 100 µl SUB (Substrat) in alle Wells.
30 Minuten bei Raumtemperatur (18-24°C) im Dunkeln inkubieren.
Pipettieren Sie 50 µl STOP (Stopplösung) in alle Wells.
Extinktion unmittelbar bei 450 nm messen, mit 630 nm als Referenz, wenn möglich.

BERECHNUNG DER ERGEBNISSE

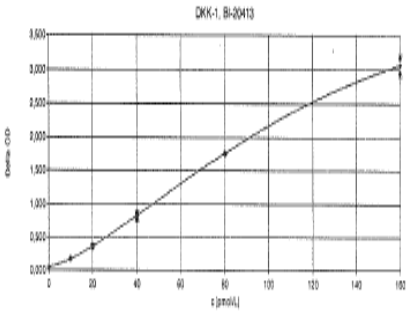
Messen Sie die optische Dichte (OD) von allen Wells mit einem Mikrotiterplattenphotometer mit einem 450 nm
er (Referenz 630 nm). Die OD des Leerwertes ist von den Werten der STD, CTRL und Proben abzuziehen.
nstruieren Sie eine Standardkurve aus den OD Werten der STD unter Verwendung von kommerziell erhältlichem
imeterpapier oder einer geeigneten Software. Das Testsystem wurde mit einem 5 Parameter Algorithmus
luiert. Andere Auswerte Algorithmen müssen vom Verwender evaluiert werden. Die Konzentration der Proben
d aus der Standardkurve abgelesen. Eventuelle Probenverdünnungen müssen bei der Berechnung der
konzentration berücksichtigt werden.

'ypische STD-Kurve:

iehe Kapitel 6) CALCULATION OF RESULTS im englischen Teil des Beipacktextes.

Das beigepackte QC Protokoll gibt die Resultate bei der QC Freigabe des Kits an. Vom Verwender erhaltene
en der OD können abweichend sein, bedingt durch verschiedene Einflüsse und/oder dem normalen
nalverlust des Kits während der Laufzeit. Dieser mögliche Signalverlust hat keinen Einfluss auf die Gültigkeit der
sultate, so lange die OD des höchsten Standards den Wert 1,50 oder höher erreicht und der Wert der CTRL im
ligen Bereich ist (Bereich siehe Etikett).

Example typical STD-curve:



The quality control (QC) protocol supplied with the kit shows the results of the final release QC for each kit at production date. Data for OD obtained by customers may differ due to various influences and/or due to the normal decrease of signal intensity during shelf life. However, this does not affect validity of results as long as an OD of 1.50 or more is obtained for the STD with the highest concentration and the value of the CTRL is in range (target range see label).

9) ASSAY CHARACTERISTICS

Method	Sandwich ELISA, 96-well strip plate, HRP/TMB			
Sample type	Serum			
Standard range	0-160 pmol/l (0, 10, 20, 40, 80, 160 pmol/l), = 0-4103 pg/ml			
Conversion factor	1 pg/ml = 0.039 pmol/l (MW = 25.8 kDa)			
Sample volume	20 µl human serum			
Incubation time, temp.	DAY TEST 2 h / 1 h / 30 min, room temperature (18-24°C)			
Sensitivity	LOD: 1.7 pmol/l (0 pmol/l + 3 SD) , LLOQ: 1.25 pmol/			
Specificity	oligomeric forms of natural and recombinant human Dkk-1.			
Cross-reactivity	Human only, No cross-reactivity or interference with recombinant human Dkk-4, Kremen-1, Kremen-2 or LRP-6 is observed.			
Precision	Intra-assay (n=5) ≤ 3% , Inter-assay (n=9) ≤ 3%			
Spike/Recovery (average recovery spiked with 40 pmol/l rec. DKK-1)	Serum (n=8)= 92%			
Dilution linearity (average recovery of expected DKK-1 after a 1+1; 1+3; 1+7 dilution)	Dilution:	1+1	1+3	1+7
	Serum (n=4)	109%	104%	109%
Values from apparently healthy individuals	Median Serum (n=51)= 34 pmol/l Each laboratory should establish its own reference range for the samples under investigation.			

For further information on assay characteristics please visit our website www.bmgrp.com/ technical file or contact our customer service by e-mail export@bmgrp.com or by phone +43/ 1/ 29107-45.

Follicle Stimulating Hormone (FSH) ELISA

For the quantitative determination of FSH in human serum

For "*In Vitro* Diagnostic" use within the United States of America.
This product is for "Research Use Only" outside of the United States of America.

Catalog Number:	11-FSHHU-E01
Size:	96 wells
Version:	9.1 - ALPCO 05Nov13

26-G Keewaydin Drive, Salem, NH 03079 | P: (800) 592-5726 | F: (603) 898-6854 | ts@alpco.com | www.alpco.com

For in vitro diagnostic use only.

PRINCIPLE OF THE TEST

The principle of the following enzyme immunoassay test follows a typical two-step capture or „sandwich" type assay. The assay makes use of two highly specific monoclonal antibodies: A monoclonal antibody specific for FSH is immobilized onto the microwell plate and another monoclonal antibody specific for a different region of FSH is conjugated to horse radish peroxidase (HRP). FSH from the sample and standards are allowed to bind to the plate, washed, and subsequently incubated with the HRP conjugate. After a second washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stop solution. The absorbance is measured on a microtiter plate reader. The intensity of the color formed by the enzymatic reaction is directly proportional to the concentration of FSH in the sample.

A set of standards is used to plot a standard curve from which the amount of FSH in patient samples and controls can be directly read.

CLINICAL APPLICATIONS

Human follicle stimulating hormone (FSH) is a glycoprotein hormone produced by the anterior pituitary gland. There are three other glycoprotein hormones, namely Thyroid Stimulating Hormone, Luteinizing Hormone (both produced by anterior pituitary gland) and Human Chorionic Gonadotropin (produced by the placenta) which are structurally similar. Each hormone has an alpha and beta subunit. The α subunits of each hormone are similar while the β subunit is specific to each hormone. The α subunits contain 92 amino acids while the β subunits vary with each hormone. The β subunit of both FSH and LH contain 115 amino acids, TSH 110 amino acids, and hCG 147 amino acids.

The FSH and LH hormones function differently in females and males. It is to be noted that in women the growth and maturation of the ovarian follicle is dependent on FSH, while in men both LH and FSH act on the testes.

PROCEDURAL CAUTIONS AND WARNINGS

1. Users should have a thorough understanding of this protocol for the successful use of this kit. Reliable performance will only be attained by strict and careful adherence to the instructions provided.
2. Control materials or serum pools should be included in every run at a high and low level for assessing the reliability of results.
3. When the use of water is specified for dilution or reconstitution, use deionized or distilled water.
4. In order to reduce exposure to potentially harmful substances, gloves should be worn when handling kit reagents and human specimens.
5. All kit reagents and specimens should be brought to room temperature and mixed gently but thoroughly before use. Avoid repeated freezing and thawing of reagents and specimens.
6. A calibrator curve must be established for every run.
7. The controls should be included in every run and fall within established confidence limits.
8. Improper procedural techniques, imprecise pipetting, incomplete washing as well as improper reagent storage may be indicated when assay values for the controls do not reflect established ranges.
9. When reading the microplate, the presence of bubbles in the microwells will affect the optical densities (ODs). Carefully remove any bubbles before performing the reading step.
10. The substrate solution (TMB) is sensitive to light and should remain colorless if properly stored. Instability or contamination may be indicated by the development of a blue color, in which case it should not be used.
11. When dispensing the substrate and stop solution, do not use pipettes in which these liquids will come into contact with any metal parts.
12. To prevent contamination of reagents, use a new disposable pipette tip for dispensing each reagent, sample, standard and control.
13. Do not mix various lot numbers of kit components within a test and do not use any component beyond the expiry date printed on the label.
14. Kit reagents must be regarded as hazardous waste and disposed of according to national regulations.

LIMITATIONS

1. All the reagents within the kit are calibrated for the direct determination of FSH in human serum. The kit is not calibrated for the determination of FSH in saliva, plasma or other specimens of human or animal origin.
2. Do not use grossly hemolyzed, grossly lipemic, icteric or improperly stored serum.
3. Any samples or control sera containing azide or thimerosal are not compatible with this kit, as they may lead to false results.
4. Only calibrator A may be used to dilute any high serum samples. The use of any other reagent may lead to false results.
5. The results obtained with this kit should never be used as the sole basis for clinical diagnosis. For example, the occurrence of heterophilic antibodies in patients regularly exposed to animals or animal products has the potential of causing interferences in immunological tests. Consequently, the clinical diagnosis should include all aspects of a patient"s background including the frequency of exposure to animals/products if false results are suspected.
6. Some individuals may have antibodies to mouse protein that can possibly interfere in this assay. Therefore, the results from any patients who have received preparation of mouse antibodies for diagnosis or therapy should be interpreted with caution.

SAFETY CAUTIONS AND WARNINGS

POTENTIAL BIOHAZARDOUS MATERIAL

Human serum that may be used in the preparation of the standards and controls has been tested and found to be non-reactive for Hepatitis B surface antigen and has also been tested for the presence of antibodies to HCV and Human Immunodeficiency Virus (HIV) and found to be negative. However no test method can offer complete assurance that HIV, HCV and Hepatitis B virus or any infectious agents are absent. The reagents should be considered a potential biohazard and handled with the same precautions as applied to any blood specimen.

CHEMICAL HAZARDS

Avoid contact with reagents containing TMB, hydrogen peroxide and sulfuric acid. If contacted with any of these reagents, wash with plenty of water. TMB is a suspected carcinogen.

SPECIMEN COLLECTION AND STORAGE

Approximately 0.1 ml of serum is required per duplicate determination. Collect 4-5 ml of blood into an appropriately labeled tube and allow it to clot. Centrifuge and carefully remove the serum layer. Store at 4°C for up to 24 hours or at -10°C or lower if the analyses are to be done at a later date. Consider all human specimens as possible biohazardous materials and take appropriate precautions when handling.

SPECIMEN PRETREATMENT

This assay is a direct system; no specimen pretreatment is necessary.

REAGENTS AND EQUIPMENT NEEDED BUT NOT PROVIDED

1. Precision pipettes to dispense 25, 50, 100 and 300 µl
2. Disposable pipette tips
3. Distilled or deionized water
4. Plate shaker
5. Microwell plate reader with a filter set at 450nm and an upper OD limit of 3.0 or greater* (see assay procedure step 13)
6. Centrifuge

REAGENTS PROVIDED

1. Mouse Anti-FSH Antibody Coated Microwell Plate-Break Apart Wells - Ready To Use.
Contents: One 96 well (12x8) monoclonal antibody-coated microwell plate in a resealable pouch with desiccant.
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.

2. Mouse Anti-FSH Antibody-Horseradish Peroxidase (HRP) Conjugate - Requires Preparation.
Contents: Anti-FSH monoclonal antibody-HRP conjugate in a protein-based buffer with a non-mercury preservative.
Volume: 300 µl/vial
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.
Preparation: Dilute 1:50 in assay buffer before use (eg. 40 µl of HRP in 2 ml of assay buffer). If the whole plate is to be used dilute 240 µl of HRP in 12 ml of assay buffer. Discard any that is left over.

3. FSH Calibrators - Ready To Use.
Contents: Six vials containing FSH in a protein-based buffer with a non-mercury preservative. Prepared by spiking buffer with a defined quantity of FSH. Calibrated against World Health Organization (WHO) 1st IS 83/575.
*Listed below are approximate concentrations, please refer to vial labels for exact concentrations.

Calibrator	Concentration	Volume
Calibrator A	0 IU/L	2.0 ml
Calibrator B	5 IU/L	0.5 ml
Calibrator C	10 IU/L	0.5 ml
Calibrator D	20 IU/L	0.5 ml
Calibrator E	50 IU/L	0.5 ml
Calibrator F	100 IU/L	0.5 ml

Storage: Refrigerate at 2-8°C
Stability: 12 months in unopened vials or as indicated on label. Once opened, the standards should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

4. Controls - Ready To Use.
Contents: Two vials containing FSH in a protein-based buffer with a non-mercury preservative. Prepared by spiking serum with defined quantities of FSH. Refer to vial labels for the acceptable range.
Volume: 0.5 ml/vial
Storage: Refrigerate at 2-8°C
Stability: 12 months in unopened vial or as indicated on label. Once opened, the controls should be used within 14 days or aliquoted and stored frozen. Avoid multiple freezing and thawing cycles.

5. Wash Buffer Concentrate - Requires Preparation.
Contents: One bottle containing buffer with a non-ionic detergent and a non-mercury preservative.
Volume: 50 ml/bottle
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.
Preparation: Dilute 1:10 in distilled or deionized water before use. If the whole plate is to be used dilute 50 ml of the wash buffer concentrate in 450 ml of water.

6. Assay Buffer - Ready To Use.
Contents: One vial containing a protein-based buffer with a non-mercury preservative.
Volume: 25 ml/vial
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.

7. TMB Substrate - Ready To Use.
Contents: One bottle containing tetramethylbenzidine and hydrogen peroxide in a non-DMF or DMSO containing buffer.
Volume: 16 ml/bottle
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.

8. Stop Solution - Ready To Use.
Contents: One vial containing 1M sulfuric acid.
Volume: 6 ml/vial
Storage: Refrigerate at 2-8°C
Stability: 12 months or as indicated on label.

ASSAY PROCEDURE

Specimen Pretreatment: ***None.***

All reagents must reach room temperature before use. Calibrators, controls and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

1. Prepare working solutions of the anti-FSH-HRP conjugate and wash buffer.

2. Remove the required number of microwell strips. Reseal the bag and return any unused strips to the refrigerator.

3. Pipette 25 µl of each calibrator, control and specimen sample into correspondingly labeled wells in duplicate.

4. Pipette 100 µl of assay buffer into each well (It is recommended to use a multichannel pipette).

5. Incubate on a plate shaker (approximately 200rpm) for 30 minutes at room temperature.

6. Wash the wells 3 times with 300 µl of diluted wash buffer per well and tap the plate firmly against absorbent paper to ensure that it is dry (The use of a washer is recommended).

7. Pipette 100 µl of the conjugate working solution into each well (It is recommended to use a multichannel pipette).

8. Incubate on a plate shaker (approximately 200 rpm) for 30 minutes at room temperature.

9. Wash the wells again in the same manner as step 6.

10. Pipette 100 µl of TMB substrate into each well at timed intervals.

11. Incubate on a plate shaker for 15-20 minutes at room temperature (or until calibrator F attains dark blue color for desired OD).

12. Pipette 50 µl of stop solution into each well at the same timed intervals as in step 10.

13. Read the plate on a microwell plate reader at 450 nm within 20 minutes after addition of the stop solution.

* If the OD exceeds the upper limit of detection or if a 450 nm filter is unavailable, a 405 or 415 nm filter may be substituted. The optical densities will be lower, however, this will not affect the results of patient/control samples.

CALCULATIONS

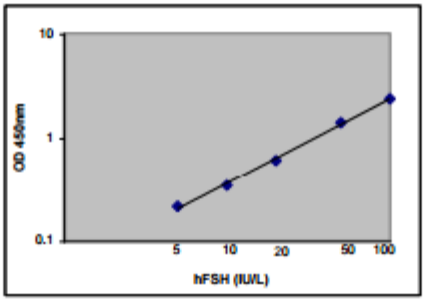
1. Calculate the mean optical density of each calibrator duplicate.
2. Calculate the mean optical density of each unknown duplicate.
3. Subtract the mean absorbance value of the "0" calibrator from the mean absorbance values of the calibrators, controls and serum samples.
4. Draw a calibrator curve on log-log paper with the mean optical densities on the Y-axis and the calibrator concentrations on the X-axis. If immunoassay software is being used, a 4-parameter or 5-parameter curve is recommended.
5. Read the values of the unknowns directly off the calibrator curve.
6. If a sample reads more than 100 IU/L then dilute it with calibrator A at a dilution of no more than 1:8. The result obtained should be multiplied by the dilution factor.

TYPICAL TABULATED DATA

Calibrator	OD 1	OD 2	Mean OD	Value (IU/L)
A	0.073	0.071	0.072	0
B	0.218	0.209	0.214	5
C	0.336	0.347	0.342	10
D	0.603	0.601	0.602	20
E	1.433	1.360	1.397	50
F	2.374	2.370	2.372	100
Unknown	0.269	0.263	0.266	7.2

TYPICAL CALIBRATOR CURVE

Sample curve only. Do not use to calculate results.



PERFORMANCE CHARACTERISTICS

SENSITIVITY

The lower detection limit is calculated from the standard curve by determining the resulting concentration of the mean OD of Calibrator A (based on 10 replicate analyses) plus 2 SD. Therefore, the sensitivity of the Direct FSH ELISA kit is **1 IU/L.**

SPECIFICITY (CROSS REACTIVITY)

The specificity of the Direct hFSH ELISA kit was determined by measuring the apparent hFSH value of calibrator A spiked with the following compounds:

Substance	Concentration Range	Apparent hFSH Value (IU/L)
hCG Calibrated against WHO 3rd IS 75/537	1000-50,000 IU/L	Not Detected
hLH Calibrated against WHO 2nd IS 80/552	5-250 IU/L	Not Detected
hTSH Calibrated against WHO 2nd IS 80/558	5-250 mIU/L	<4.0

INTRA-ASSAY PRECISION

Three samples were assayed ten times each on the same calibrator curve. The results (in IU/L) are tabulated below:

Sample	Mean	SD	CV%
1	7.41	0.43	5.8
2	48.57	1.70	3.5
3	138.12	4.70	3.4

INTER-ASSAY PRECISION

Three samples were assayed ten times over a period of four weeks. The results (in IU/L) are tabulated below:

Sample	Mean	SD	CV%
1	7.11	0.24	3.4
2	44.31	2.01	4.5
3	120.63	7.74	7.7

Sample	Obs.Result	Exp.Result	Recovery%
1	5.65	-	-
Unspiked	14.75	15.35	96.1
+9.7	56.68	59.15	95.8
+53.5	103.33	112.65	91.7
+107.0			
2	17.52	-	-
Unspiked	26.21	27.22	96.3
+9.7	70.07	71.02	98.7
+53.5	116.40	124.52	93.5
+107.0			
3	58.47	-	-
Unspiked	72.71	68.17	106.7
+9.7	114.25	111.97	102.0
+53.5	171.05	165.47	103.4
+107.0			

LINEARITY

Three patient serum samples were diluted with calibrator A. The results (in IU/L) are tabulated below:

Sample	Obs.Result	Exp.Result	Recovery%
1	22.56	-	-
1:2	11.89	11.28	105.4
1:4	6.47	5.64	114.7
1:8	3.27	2.82	116.0
2	123.42	-	-
1:2	66.80	61.71	108.2
1:4	31.78	30.86	103.0
1:8	17.05	15.43	110.5
3	162.67	-	-
1:2	77.93	81.34	95.8
1:4	39.35	40.67	96.8
1:8	21.86	20.33	107.5

HIGH DOSE HOOK EFFECT

The Direct hFSH ELISA kit did not experience any high dose hook effect when tested up to a FSH concentration of 50,000 IU/L.

REFERENCE VALUES

As for all clinical assays each laboratory should collect data and establish their own range of expected normal values.

Group	Range (IU/L)
Males	1-18
Females	
Follicular Stage	2-10
Midcycle Peak	7-20
Luteal Stage	1-10
Postmenopausal	18-150

REFERENCES

1. Butt, W.R. et al., J. Endocrinol. 58:275, 1973.
2. Kjeld, J.M., et., al., Acta Endocrinol. 81:225, 1976.
3. Marshall, J.C., et al., J. Endocrinol. 56:431, 1973.
4. Seth J., et al., Clin. Chim. Acta. 186:67, 1989.
5. Jockenhovel F., et al., J. Clin. Chem. Clin. Biochem 27:825, 1989.
6. Shome B.,et al.,J. Clin. Endo. Metal. 39:199, 1974.
7. Shome B., et al., J. Clin. Endo. Metal. 39:203, 1974.
8. Labrie F., et al., In Karolinska Symposium on Research method in Reproductive Endocrinology (Diczfalusy,E,ed) Acta Endocrinol. supl. 180:301, 1973.
9. Knobil, E., Rec. Prog. Horm. Res. 36:53, 1980.