UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

A LONGITUDINAL ASSESSMENT OF BIOMARKERS, MUSCULAR PERFORMANCE, AND AEROBIC CAPACITY IN COLLEGE-AGED ROTC MEMBERS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

Degree of

DOCTOR OF PHILOSOPHY

By

BREANNE SLOAN BAKER
Norman, Oklahoma
2018

A LONGITUDINAL ASSESSMENT OF BIOMARKERS, MUSCULAR PERFORMANCE, AND AEROBIC CAPACITY IN COLLEGE-AGED ROTC MEMBERS

A DISSERTATION APPROVED FOR THE
DEPARTMENT OF HEALTH AND EXERCISE SCIENCE

BY

_____
Dr. Debra A. Bemben, Chair

_____
Dr. Michael G. Bemben

_____
Dr. Christopher Black

_____
Dr. Allen Knehans

_____
Dr. Rosemary Knapp

Acknowledgements

I am a product of the many mentors, friends, and family members who have taken the time and effort to pour into my life. This accomplishment signifies as many of their sacrifices as it does mine, and I am forever indebted for their commitment and encouragement. Both Dr. Mike Bemben and Dr. Chris Black shared lab space and equipment for data collection, which allowed for the expansion of physical performance testing. Dr. Knehans and Dr. Knapp have provided excellent feedback and support throughout this process. Cameron, Kelsey, Michelle, Alison, and Sam, played integral roles for each aspect of testing and without even one of their efforts, this longitudinal study would not have been successful. I am indebted to the participants who always arrived with a smile on their faces and sacrificed their time, sweat, and blood.

Lastly, and most importantly Dr. Debra Bemben has provided countless hours of mentorship to build me as a student, researcher, and person. Without her constant guidance, this research would not have been possible, and I would have not pursued this degree. I will model much of my future career in academia based on how she has served as a mentor who was demanding and tough, and never compromised her standards of excellence.

Table of Contents

List of Tables

List of Figures

Abstract

Military personnel engage in vigorous PA, which would suggest higher bone mineral density (BMD) and lower fracture incidence rates; however, bone injuries are common in this cohort. Imaging devices such as DXA and pQCT scanners have been used in addition to serum bone turnover markers (BTM) to describe skeletal responses to military training interventions; however, researchers are investigating novel biomarkers due to poor injury prediction capabilities of BTM. Parathyroid Hormone (PTH) and sclerostin are two markers that have been predictive of fracture in other populations. PTH can cause increased bone resorption and it is a strong predictor of fracture risk in postmenopausal women; however, the investigation of this marker in military populations has produced confounding results. Sclerostin is secreted by osteocytes and is also a marker of bone resorption; however, to date very few studies have investigated the marker's response to longitudinal exercise in humans. **Purpose:** The purpose of this study was to determine the effects of an eight week military training intervention on PTH and sclerostin serum concentrations, and aBMD of the total body, dual femur, and lumbar spine, and bone geometry of the tibia, in healthy, college-aged USMC and Naval Reserve Officers' Training Corps (ROTC) members as compared to a matched control group. A secondary purpose was to determine the relationship between PTH and sclerostin and bone variables. Lastly, group differences in body composition, upper and lower body muscular strength and power measures, and aerobic fitness and their relationships to PTH and sclerostin serum concentrations were examined. **Methods:** Eighteen college-aged ROTC members were matched for sex, age (±2yrs), and body mass (±5lbs) to physically active controls. ROTC participants engaged in an eight week

training intervention, while controls made no changes to their physical activity. Total body areal BMD (aBMD) and body composition were measured by DXA at the pre, mid, and post testing periods. Regional aBMD of the lumbar spine and hips were measured by DXA at the pre and post testing periods. Volumetric BMD (vBMD) of the non-dominant tibia was measured by pQCT at the pre and post testing periods. Serum PTH and sclerostin were assessed from pre and post blood draw using commercial ELISA kits. Lastly, measures of muscular strength and power were tested at the pre, mid, and post testing periods while aerobic capacity was tested pre and post intervention. **Results:** Both groups decreased total body and regional fat mass (all $p \leq 0.047$) while only ROTC participants exhibited significant increases in dominant femoral neck, and dominant total hip aBMD and BMC (all $p \leq 0.033$) after the eight week intervention. No consistent group or time differences were found for pQCT variables or biomarker responses. ROTC members started and ended the intervention with greater relative VO_2 peak measures and also increased relative $VO_{2\ peak}$ significantly more than controls (time effect $p=0.007$; group effect $p=0.014$). At both time points serum sclerostin demonstrated strong positive correlations with aBMD, vBMD, and performance measures in the ROTC group. **Conclusion:** An eight week military training intervention did not result in skeletal changes suggestive of increased risk for injury as compared to a matched control group. In fact the intervention resulted in greater aBMD of the lumbar spine and hip regions in ROTC members. Serum biomarker responses were not significantly different over time; however, sclerostin may provide additional information regarding skeletal changes in this cohort.

Chapter 1: Introduction

Bone turnover is a dynamic process in which the skeletal tissue responds to stimuli in order to meet the body's demands for structural integrity, protection, and minerals. Physical activity (PA) is often viewed as osteogenic because muscle contractions and vertical ground reaction forces load the bone, resulting in microdamage, which initially signals bone resorption, followed by reparative bone formation (1-3). If the timing between vigorous and potentially damaging PA bouts is too short and does not allow for adequate bone formation to occur, it may reduce the integrity of the bone and increase the risk for injury (4). One of the most common bone injuries is a fracture, which includes osteoporotic and stress fractures. Osteoporotic fractures are primarily due to reduced mineral matrix, resulting in poor integrity of the bone (5); while stress fractures are characterized as cumulative microdamage or trauma to the bone, resulting in pain and reduced loading capabilities (6).

Athletes from a wide variety of disciplines present with stress fractures; however, tactical athletes are of special concern. The term tactical athlete refers to law enforcement, military, and rescue professionals who require unique parameters of physical fitness and technical skills (7). Military personnel engage in vigorous PA, which would suggest higher bone mineral density (BMD) and lower fracture incidence rates; however, bone injuries are common in this cohort (8-11). According to the U.S. Government Accountability Office, bone injury is a significant contributor to attrition rates within the first six months of military service (12). Even recruits who fully recover from these injuries are at significantly greater risk for another fracture during subsequent service (10.6% incidence within one year of injury, versus 1.7% in injury-

free recruits), which poses a financial burden and combat risk (13). The Department of Defense estimates that bone injuries in military personnel cost over $100 million annually in medical care and lost productivity (14). Studies conducted on American, Israeli, and Finnish army recruits reported that fracture incidence rates during basic training range from 7-31% (15-17).

The two most common imaging techniques used in the assessment of bone quality are dual-energy x-ray absorptiometry (DXA) and peripheral quantitative computed tomography (pQCT) (18). Beck et al. (19) used both DXA and pQCT to describe the differences between male and female military recruits who suffered fractures. Recruits who presented with fractures had poorer physical fitness, smaller muscle cross-sectional area (mCSA) and bone strength indices of the thigh and tibia compared to those military recruits who did not report fractures (19). In female Naval Academy recruits, low BMD, as measured by DXA, was strongly correlated with fracture risk during eight weeks of training (8). These imaging methods provide valuable assessments of bone density and quality.

Bone metabolism at the cellular level, however, can be better understood using serum bone turnover markers (BTM). BTM are circulating biomarkers that are commonly used in conjunction with imaging devices to better describe acute and chronic skeletal adaptations (20). BTM have been identified for both resorption and formation processes and can be assayed from small samples of serum. For example, biomarkers such as procollagen type I N propeptide (PINP) and bone-specific alkaline phosphatase (bone ALP) are commonly used as markers of bone formation, while C-telopeptide of type I collagen (CTX-I) and tartrate-resistant acid phosphatase 5b

(TRAP5b) are used to describe bone resorption (21). PINP is a class of peptides that is specific to proliferating osteoblasts, the cells responsible for building bone (22), and bone ALP is an enzyme produced by osteoblasts that aids osteoid formation and mineralization (23). When osteoclasts resorb bone, a variety of proteins are released; however, 90% of these proteins are fragments of type I collagen. CTX-I is a product of type I bone collagen degradation and is used as a bone resorption marker (24). An additional bone resorption marker used is TRAP5b. TRAP5b is secreted from the osteoclasts ruffled edge during migration from one resorption pit to another, and is used as a marker of mature osteoclast number (25).

Several studies have identified BTM as strong correlates of stress fracture in clinical populations (26, 27); however, the evidence for BTM predictive power is inconsistent within athletic and military populations. Bennell et al. (28) followed nearly 100 track and field athletes for 12 months to evaluate the role of BTM in the pathogenesis of stress fractures. Those who suffered fractures did not have different serum concentrations of BTM at any point during the 12 months compared to those who remained injury free (29). Prospective studies have followed military members throughout training periods and found that BTM were strong predictors of injury status (30), while other prospective studies demonstrated no relationship (3, 17, 31). Inconsistencies in these results might stem from the heterogeneity of sex, age, and type of military training in each of the populations that were followed.

Novel biomarkers need to be investigated for potential viability as sensitive injury predictors in addition to the use of imaging machines and BTM. Parathyroid hormone (PTH) is being investigated as an endocrine regulator of bone in military

cohorts. PTH is a major mediator of serum calcium ion concentrations, with target tissues being the kidneys and bone (32). PTH is secreted by the parathyroid glands in response to calcium-sensing receptors detecting decreases in serum calcium ion concentrations (33). In bone, PTH stimulates osteoclasts to resorb bone mineral, increasing serum calcium ion concentrations. In the kidneys, PTH signals the increase in proximal tubular resorption of calcium and formation of vitamin D, while simultaneously increasing the amount of phosphate excreted in an attempt to restore calcium homeostasis (32). Prospective studies have found that PTH is chronically elevated post basic training in male (17) and female (34) military recruits, while it did not change in elite male combat trainees (31), and it decreased in male and female recruits over a four-month basic training period (35).

Sclerostin is another biomarker that is being used to describe bone metabolism. Sclerostin is a glycoprotein secreted from osteocytes and acts as a negative regulator of bone formation via Wnt signaling inhibition (36, 37). The Sclerostin (SOST) gene product is used by osteocytes to fine tune the skeletal response to mechanical loading (38). Animal models have shown that with increased mechanical loading sclerostin production is reduced (39). In humans, many cross-sectional studies have demonstrated acute increases in sclerostin post exercise; however, many of these studies do not account for plasma volume shifts which could result in inaccurate calculations of the sclerostin response (38, 40). The few longitudinal studies that exist demonstrate that with increased exercise, sclerostin concentrations decrease, much like the animal data suggests (41, 42). In postmenopausal women, studies have shown with regression analysis that higher concentrations of sclerostin are associated with increased fracture

risk; however, its usage in the description of military personnel bone health is currently nonexistent (43, 44).

Body composition and fitness levels also play a role in bone injury for military personnel. The negative correlation between muscle mass and fracture risk is well documented in a variety of populations (45-47). Naval Academy students who presented with fractures during basic training exhibited four times more body mass loss, had lower total body bone mineral content (BMC), and thigh mCSA compared to those students who remained injury free (8). Protective factors for bone overuse injuries include aerobic fitness (48), lower body mCSA (49), and performing weight-bearing exercises (50). Newly implemented training regimens that combine aerobic fitness and muscular strength/power have been shown to be an important tool in the reduction of military fracture rates, especially in the United States Marine Corps (USMC) and Navy (6, 50, 51). The exact relationships between aerobic fitness and muscular strength/power and biomarkers used for fracture prediction in military populations is understudied.

Purpose

The purpose of this study was to determine the effects of an eight week military training intervention on PTH and sclerostin serum concentrations, and areal bone mineral density (aBMD) of the total body, dual femur, and lumbar spine, and bone geometry of the tibia, in healthy, college-aged USMC and Naval Reserve Officers' Training Corps (ROTC) members as compared to a matched control group. A secondary purpose was to determine the relationship between PTH and sclerostin and bone variables. Lastly, group differences in body composition, upper and lower body

muscular strength and power measures, and aerobic fitness and their relationships to PTH and sclerostin serum concentrations were examined. ROTC members ranged 18-29 years old and all completed mandatory group training sessions that included endurance and resistance exercises.

Research Questions

1. Will an eight week military training intervention period (MTIP) significantly alter the PTH and sclerostin serum concentrations, and will these responses be different from those in a physically active age- and body-mass matched control group who does not participate in the MTIP?
2. Will an eight week MTIP result in significant total body aBMD changes and site specific aBMD changes at the lumbar spine and dual femur, and will these changes be different from those in a physically active age- and body-mass matched control group who does not participate in the MTIP?
3. Will an eight week MTIP alter bone content, geometry, and strength of the 4%, 38%, 66% non-dominant tibia sites, and will these changes be different from those in a physically active age- and body-mass matched control group who does not participate in the MTIP?

Hypotheses

1. PTH serum concentrations will significantly increase, while serum sclerostin concentrations will decrease in ROTC members but not in controls.
2. Total body and site specific aBMD will significantly change in ROTC members but not in controls.

3. Significant skeletal geometry changes of the 4%, 38%, 66% non-dominant tibia sites will occur in ROTC members but not in controls.

Sub Questions

1. Will there be significant relationships between serum PTH and sclerostin and total body and site-specific aBMD, bone free lean body mass (BFLBM) and fat mass (FM)?
2. Will there be significant relationships between serum PTH and sclerostin and 4%, 38%, 66% non-dominant tibia sites measures of bone strength and geometry?
3. Will there be significant relationships between serum PTH and sclerostin and muscular power and strength?
4. Will there be significant relationships between serum PTH and sclerostin and aerobic capacity?

Sub Hypotheses

1. ROTC members will demonstrate significant increases in aBMD, BFLBM and a significant decrease in FM as compared to controls, and these changes will have significant relationships with biomarkers. aBMD and BFLBM will be positively correlated and FM and serum PTH concentrations and will be negatively correlated with serum sclerostin concentrations.
2. ROTC members will demonstrate significant increases in measures of bone strength and quality at the 4%, 38%, 66% non-dominant tibia sites and these changes will have significant negative correlations with PTH and sclerostin.

3. ROTC members will demonstrate significant increases in muscular strength and power measures as compared to controls, and these changes will have significant positive correlations with PTH and negative correlations with sclerostin.
4. ROTC members will demonstrate significant increases in aerobic fitness as compared to controls, and these changes will have significant positive correlations with PTH and negative correlations with sclerostin.

Significance of the Study

Military personnel who sustain a stress fracture during training are removed from training for an average of 62 days (52). In fact, during Marine Corps basic training, the single most powerful predictor of discharge is a stress fracture, with a four-fold increased rate of discharge in soldiers who suffered from a stress fracture as compared to their counterparts who remain fracture free (53). Researchers in this area suggest that the current methods of fracture prediction in this population are severely lacking, resulting in an underestimation of the impact of stress fractures in military populations; thus there is a need for investigation of additional biomarkers to provide more predictive power (51). PTH and sclerostin may provide new information to clinicians regarding fracture etiology in military populations. This study provided scientific evidence for how these biomarkers and bone responded to an eight week MTIP in college-aged USMC and Naval ROTC members as compared to controls. Additionally, this investigation characterized the relationship between these biomarkers, and parameters of bone health, body composition, muscular strength and power, and aerobic fitness.

Assumptions

1. All participants gave maximal effort during the strength and aerobic capacity testing sessions.
2. All participants provided accurate and truthful information for all questionnaires.
3. All participants were fasted for at least eight hours and rested at least 24 hours prior to blood draws.

Delimitations

1. The findings of this study are applicable only to healthy, college-aged, ROTC members who undergo the same military training periods and their matched controls.
2. The participants were recruited only from the University of Oklahoma.

Limitations

1. Nutritional status and prescreening fitness levels were not controlled; however, calcium intake was measured at the pre testing period.
2. Unstructured physical activity was not controlled, but was quantified by questionnaires for both ROTC members and controls.
3. All ROTC members completed the same exercises but the load, repetitions, and relative intensities were not uniform.

Operational Definitions

Areal Bone Mineral Density (aBMD): aBMD (g/cm^2) is calculated as bone mineral content (g) divided by bone area (cm^2) as measured by DXA (54).

Body Composition: Is a mathematical representation of tissue types within the human body: normally expressed relative to body mass, such as percent fat (55).

Bone Mineral Content (BMC): DXA-derived BMC values refer to the amount of mineral (g) within a specified region of analysis. pQCT-derived BMC values refer to the amount of mineral per unit of axial bone length (mg/mm) (56).

Bone Remodeling: An integrated process where different types of specialized bone cells coordinate the resorption and formation of new bone, also referred to as bone metabolism (57). Bone resorption is the process of degrading skeletal matrix, primarily performed by osteoclasts. Bone formation is the process of binding new skeletal matrix, primarily performed by osteoblasts (57).

Bone Strength Index (BSI): BSI is the product of cross-sectional moments of inertia (mm^4) and cortical volumetric density (mg/mm^3) (58).

Bone Turnover Makers (BTM): Are circulating biomarkers that are commonly used to describe bone cell activity resulting in either bone formation or resorption (20, 59).

Cortical Area: The area of pixels identified as cortical by the pQCT (mm^2) (58).

Cortical Bone: The primary structure that provides rigidity to the skeleton. It is dense bone and located toward the outer part of the bone ending at the periosteum (60).

Cortical Thickness: is the thickness of all pixels identified as cortical bone by the pQCT software (mm) (61).

Counter Movement Jump: Is a functional jumping task in which a squat to a self-selected depth is performed prior to the explosive vertical jumping movement is competed (230).

Dual Energy X-Ray Absorptiometry (DXA): DXA uses the attenuation of x-rays through tissues to measure the composition of that tissue. This type of measurement is areal, only providing a two-dimensional representation of the scanned area. DXA scanners can measure total body and site-specific bone mass, fat mass, and bone-free lean body mass (54).

Endosteal Circumference: is the thin inner membrane that surrounds the medullary cavity and is measured by distance (mm) (61).

Muscle Cross-Sectional Area (mCSA): is the total area of a muscle (mm^2) (55).

One-Repetition Maximum Effort: (1RM) is the maximal amount of external load ones musculoskeletal system can overcome (231).

Osteoblast: A specialized bone surface cell responsible for bone formation (59).

Osteoclast: A specialized bone surface cell responsible for bone resorption (59).

Osteocyte: A specialized mechanosensitive bone cell embedded within the mineral matrix, primarily responsible for signaling the skeletal tissue through an extensive lacunocanalicular network of dendritic processes (59).

Osteopenia: is a condition of lower than normal BMD indicated by a T-Score of -1.1 to -2.4 as measured by DXA, in adults 50 years or older. In males and females younger than 50 years, Z-scores are used to classify bone status instead of T-scores. A Z-score ≤ −2.0 is defined as having a BMD value below the expected range for age (62).

Osteoporosis: is a condition of low BMD resulting in reduced bone strength and increased risk of bone injury. It is normally indicated by a lumbar spine, total hip, or femoral neck T-Score of ≤ -2.5, as measured by DXA in adults 50 years or older (62).

Parathyroid Hormone (PTH): is a peptide hormone secreted from the parathyroid glands in response to calcium-sensing receptors detecting decreases in plasma ionized calcium concentrations and plays a pivotal role in calcium homeostasis and subsequently the bone remodeling process (63).

Peripheral Quantitative Computed Tomography (pQCT): pQCT uses the attenuation of x-rays through tissues to measure the composition of that tissue. This type of measurement is volumetric due to multiple slices being combined (mg/cm^3) and can provide a determination of cortical and trabecular bone in addition to mCSA (54).

Periosteal Circumference: is the thin outer membrane that surrounds the cortical shell and is measured by distance (mm) (61).

Polar Moment of Inertia (iPolar): iPolar estimates the ability of the bone structure to withstand torsional forces (cm^4) (56).

Regular Menstrual Cycle: A recurring menstrual cycle without more than three consecutive months of disruption (64).

Sclerostin: is a glycoprotein secreted by osteocytes and a SOST gene product, which acts as a negative regulator of bone formation via Wnt signaling inhibition (36).

Stress Fracture: is cumulative microdamage or trauma due to repetitive loading coupled with insufficient rest periods, resulting in impaired structure integrity (6).

Stress-Strain Index (SSI): SSI combines both measures of bone geometry (section modulus) and quality (cortical vBMD (mm^3)) to provide a more comprehensive measure of bone integrity (58).

T-Score: is the number of standard deviations above or below the mean BMD for the young Caucasian female reference population (65).

Trabecular Bone: Also referred to as cancellous bone, is a spongy mineral matrix with large surface area and metabolic activity. It is located towards the inner part of bones and near the distal ends of long bones (60).

Maximal Volume of Oxygen Consumption: Maximal volume of oxygen (VO_2 max) consumption refers to the maximal volume of oxygen being consumed during exercise. VO_2 max is defined at the maximum amount of oxygen that can be consumed during maximal effort exercise divided by kg of body mass per minute; while VO_2 peak is defined as the two greatest reported VO_2 values within 30 seconds of each other during a maximal effort exercise divided by kg of body mass per minute (229).

Volumetric Bone Mineral Density (vBMD): is the quotient of BMC and the total cross-sectional area of bone. Cortical vBMD and trabecular vBMD can also be measured by calculating the quotient of BMC and the total cross-sectional area of the particular bone type (mg/cm^3) (56).

Z-Score: is the number of standard deviations that a participant's aBMD is away from an age, sex, ethnicity, and body-mass matched reference population (65).

Chapter 2: Literature Review

Introduction

Having a stable bone mass requires balance between the activity of bone forming cells and bone resorbing cells, the osteoblasts and osteoclasts, respectively. A third bone cell type, osteocytes, are located in the mineralized bone tissue and can detect injury and mechanical strain (66). Many injury and disease states can be attributed to dysfunctional signaling pathways or disproportionate activity of these three cell types. For example, Van Buchem disease (VBD) is characterized by uncontrolled bone growth. The primary contributor is a dysfunctional SOST gene that cannot adequately produce sclerostin, which serves as a potent bone formation inhibitor (67). Injuries and diseases due to imbalanced bone turnover resulting in low bone mass are more common. For instance, osteoporosis is a progressive disease characterized by low bone mineral density (BMD), high rates of bone turnover, and reduced structural integrity (68). Unlike VBD, there is no single cause of osteoporosis, as genetic and dietary factors combined with physical activity (PA) and hormonal levels play vital roles in the development and progression of the disease.

Low BMD and poor bone quality reduces the bone's ability to withstand stress and increases the risk for bone injuries (69). Research has shown that pharmacological treatments and physical activity (PA) can both have positive effects on low BMD. One of the most common drug strategies is prescribing bisphosphonates, which has been shown to successfully reduce the risk of fractures; however, this class of drug is not suitable for all populations and has side effects to consider (68). PA-induced mechanical stress signals bone formation when the stimuli meet the loading profile

criteria and exceed the minimum threshold (70, 71). With loading, the skeleton sustains microdamage that is repaired, resulting in a greater strain tolerance; however, certain types of PA and loading profiles do not always allow for an adequate amount of repair. When the microdamage cannot heal in the midst of additional loading, a stress fracture may occur.

Older adults are not the only populations where low BMD and fractures are observed; many athletic and military populations also share these characteristics. Military personnel often engage in PA, which should result in higher BMD; however, bone injuries are common. Being female has been presented as the primary risk factor for bone injury in military recruits; however when age, fitness, and race are controlled, sex differences became non-significant (9). In military personnel, how the changes in bone density, geometry, strength, and injury risk are related to age, sex, dietary intake, and measures of physical performance are currently not well characterized.

Bone Physiology

Skeletal homeostasis is a dynamic and integrated process that involves a wide variety of cell types and signaling pathways. The genetic blueprint for bone is contained within the bone cells. There is also an epigenetic component of skeletal design that is directed by the chemical milieu of the cell's internal environment and the mechanical forces exerted on the bones. Together, these forces shape the bone until it can meet the loading requirements applied to it (72). There are three primary cell types that work in unison to engineer bone's structure: osteocytes, osteoblasts, and osteoclasts. The way that these cells interact also determines the spatial orientation and extent of mineralized matrix. These structural differences can be observed in the two

different types of bone, cortical and trabecular. Highly integrated signaling pathways regulate how these bone cells function throughout developmental, maintenance, and disease stages.

Osteocytes are distributed throughout the skeletal mass, connected to each other through dendritic processes, much like axons. Specifically, osteocytes make up nearly 95% of all bone cells and are an integral part of these signaling pathways and bone's mechanosensitivity. These processes usually radiate from the osteocyte towards the bone surface and towards vasculature. The small openings within the bone in which osteocytes are housed are called lacunae, while the dendrites travel through a series of tunnels called canaliculi. These cells create a mechnosensitive detection system called the lacunocanalicular network that is partially responsible for converting mechanical stimuli into chemical signals. This process is also known as mechanotransduction. Because of this particular anatomy, osteocytes have been viewed as the primary signaling cell to cue osteoblast and osteoclast activity (73).

Osteocytes are only fully functional after three distinct stages of morphology. First, osteoprogenitor cells within the bone marrow will differentiate into osteoblasts. Secondly, while functioning as osteoblasts, these bone-lining cells will transition into an osteoid-osteocyte, that has the unique capability of actively modeling matrix while also calcifying that same matrix. This cell will then shrink in size by approximately 30% and settle into a lacuna where the final transition to a mature osteocyte occurs (74).

The lacunocanalicular network utilizes each aspect of osteocyte anatomy to serve as the primary mechanosensor of the bone, which can signal both osteoclasts and osteoblasts. For instance, actin proteins act like tethers to anchor the osteocytes within

the lacunae. When fluid shifts occur, due to mechanical loading or vibration, shear forces can be sensed by these actin proteins, the primary cilium, and the osteocyte cell membrane, that will result in stimulation of osteoblast activity (74). Additionally, at the far reaches of the osteocytes' dendrites, the tips are covered in specialized gap junctions that are also sensitive to shear forces and can relay chemical messages back to the osteocyte about the mechanical forces that matrix is experiencing. Osteocytes also can signal osteoclasts to increase bone resorption in the absence of mechanical loading signals. Along the dendritic processes, NF-kappaB ligand (RANKL) expression is increased when the bone is unloaded, which signals osteoclasts to initiate the formation of resorption pits (75, 76). Traditionally, it has been thought that osteocytes only signal, and it is the osteoblasts and osteoclasts that are responsible for matrix modification. Nearly 50 years ago, it was hypothesized that osteocytes might have the capability of mineralizing their surroundings, more recently it has been shown that osteocytes can also enlarge their lacunae and canaliculi by a few angstroms (77, 78). These small alterations could enact large changes for the microarchitecture and strength of the bone because the surface area of the lacunocanalicular system is several orders of magnitude greater than the bone surface, where osteoblasts and osteoclasts have their effects (76).

It is not well characterized why osteocytes develop from their osteoblast precursor. However, the mechanisms leading from mesenchymal stem cells (MSC) to osteoblastogenesis are tightly regulated by a wide variety of factors. In fact, there are five primary cytokines that are involved with osteoblast differentiation: the Hedgehog proteins, bone morphogenetic protein (BMPs), transforming growth factor-β (TGF-β), parathyroid hormone (PTH), and Wnts in addition to the master switch for

osteoblastogenesis, Runx2. Runx2 knockout (KO) mice are not capable of producing osteoblasts and form cartilaginous skeletons that are void of mineralized matrix (79). Runx2 also targets the transcription factor Osterix (Osx). Osx KO mice also lack osteoblasts and have a many downstream pathways that are dysfunctional and often result in death (80). Runx2 cooperates with other transcription factors to control BMP-induced osteoblast gene expression and creates a positive feedback loop to regulate osteogenesis (81). While downstream targets of Runx2 also interact with TGF-β, the Hedgehog family of proteins, and Wnts, fine-tune osteoblast and osteoclast development, activity, and apoptosis (81).

Bone cells work in a coordinated manner to repair microdamage and replace old bone with new mineral deposits. Mature osteoblasts' primary purpose is to synthesize osteocalcin and osteopontin and then adhere these proteins to the exposed cross-linked collagen. Osteoblasts also play a pivotal role in mineralizing the cartilaginous skeletal during growth. Osteoclasts, on the other hand, are members of the monocyte-macrophage family from bone marrow. Two cytokines are responsible for osteoclastogenesis. The first is RANKL, and the second is macrophage-colony stimulating factor (M-CSF). RANKL is the key cytokine for osteoclast formation, while M-CSF contributes to the proliferation and survival of the osteoclast (82). Osteoclasts have a unique ability to create a microenvironment that maintains a pH of around 4.5, which is needed to release calcium phosphate and other organic compounds from the bone surface. These molecules are then further degraded by cathepsin K, before exiting the osteocyte (83, 84). The careful interaction between osteoblasts and

osteoclasts is how bone serves as the primary calcium repository in the body, and can quickly store or mobilize minerals.

Bone Metabolism

Several controllable factors have been implicated with bone status, such as diet, PA, and hormone levels; however, major uncontrollable factors such as genetic influences, age, and sex have also been identified. For instance, several twin and family studies have demonstrated that 50-85% of the variance observed in peak aBMD may be attributed to uncontrollable genetic factors (85). Age is a major confounder in skeletal health; however, the exact mechanisms are difficult to characterize due to the vast array of dysfunction that is associated with an aging individual. Similarly, being female is a factor for reduced aBMD and the differences in bone strength, geometry, and density have very strong links to sex hormones. Therefore, it is nearly impossible to target one factor without the consideration of many others.

Age-related factors in bone health can be separated into time frames of bone development and growth, maintenance, and the age-related bone loss that occurs after the fifth decade of life. Even before the age of 2 years, some skeletal traits have been established (86). Once children reach the early stages of puberty, large differences can be observed in bone geometry; however, once puberty is over, the differences in BMD are drastically reduced, suggesting that the variances observed in bone strength are more likely attributable to bone geometry instead of bone density (87). Skeletal geometry is sensitive to alterations at different time points and is site-specific. For example, the subperiosteal surface of long bones is most sensitive to alterations in mechanical loading during childhood and early adolescence, but the endocortical

surface is more mechanosensitive during puberty (88, 89). This is an issue with childhood inactivity and might affect each sex differently. How bones adapt during the developmental and growth stage can have lasting effects. For instance, the differences observed in bone size and mass around an age-specific mean is large; 1 SD is equivalent to 10-15% of the mean. The variance in the rate of bone loss is significantly smaller as 1 SD is only about 1% of the mean. This difference suggests that bone properties that are established by the end of maturity are significantly more important determinants of bone injury rates in adulthood than differences in the rates of bone loss towards the end of life (90). In 2000, Beck et al. (19) demonstrated that male and female army recruits that were age- and fracture-incidence matched, had different bone geometry. Female fracture cases had thinner cortices, while male fracture cases had smaller subperiosteal diameters, suggesting that the bone geometry developed in the early years may have been important factors in stress fracture etiology as adults.

Both men and women experience a progressive decline in BMD, which starts as soon as peak BMD is reached; therefore advancing age is a risk factor for fracture, especially for those over the age of 50 (91). As the aging process occurs, many signaling pathways and mechanisms become dysfunctional that can increase the risk for low BMD and subsequent bone injury. Reactive oxygen species (ROS), apoptosis, hormonal influences, and macroautophagy are some important factors to consider (91). ROS are responsible for the oxidation of molecules, creating oxidative stress. This oxidative stress is managed under normal conditions by a series of antioxidant scavengers; however, as the aging process continues, antioxidant activity decreases and the effects of ROS become more evident. Mice deficient in superoxide dismutase

(SOD), a potent antioxidant, present with decreased BMD, osteoblast and osteoclast numbers as compared to wild-type mice (92). The forkhead box O (FOXO) family of transcription factors are also ROS scavengers. ROS production is increased and bone loss is observed, in FOXO KO mice models. This is due to both a decreased number of osteoblasts and an impaired Wnt-β-catenin signaling pathway (93). RANKL is also upregulated with aging, resulting in increased apoptosis of osteocytes which inhibits proliferation of osteocytes and increases osteoblast apoptosis (94). Lastly, macroautophagy, or the process of recycling damaged organelles and proteins, can become dysfunctional with aging. A series of macroautophagy-related genes specific to osteoclasts, osteoblasts and osteocytes have been deleted in mice; results demonstrate drastically altered bone homeostasis (95).

Hormone concentrations begin to elicit effects on bone morphology during the prepubertal stage and continue to have pronounced effects throughout the lifespan. Although there are many hormones that are important to bone homeostasis, only three will be discussed here: estrogens, testosterone, and parathyroid hormone (PTH). Estrogen concentrations, in males and females, play a pivotal role in early bone development; however, the main estrogen of interest is estradiol (E2). Increased E2 during adolescence have been shown to dictate the amount of viable ERα, which is partially responsible for the mechanosensitivity of the bone (96, 97). Subsequently, these increased E2 levels partially explain the enhanced sensitivity to exercise and PA during the pre-pubertal stages in males and females; however, the sex-specific changes in bone geometry are not the same. During puberty, males have greater periosteal apposition, which leads to increased cortical thickness. Girls have decelerated

periosteal apposition, in addition to no changes in medullary cavity size, resulting in bone that is smaller than boys, but with a similar cortical thickness (98). High levels of E2 in post-pubertal females is thought to promote this endosteal apposition, which partly explains the higher cortical densities observed in females as compared to males (86, 99). These geometric alterations to bone, periosteal apposition specifically, result in the optimization of bone strength without adding excess material. Since males have greater periosteal apposition than females before and during puberty, it stands to reason that this is a major contributing factor to the greater bone strength observed in males across the lifespan (86).

E2 levels are drastically reduced during the menopausal transition in women. The cessation of menstruation occurs at about 48-50 years of age in most women and results in unbalanced and aggressive bone turnover, especially in trabecular bone. Indices of bone resorption are twice as high in postmenopausal women (PMPW), as compared to premenopausal women, while the bone formation markers are only 50% elevated above premenopausal levels. This skewed ratio leads to rapid bone resorption and increased risk for low BMD and bone injury (100). At the cellular level, it has been shown with a decrease in E2 levels the lifespan of osteoblasts and osteocytes decreases while the osteoclasts remain viable for longer (101). This period of accelerated bone loss lasts approximately 5-10 years and is followed by a second phase of continuous bone loss. Hormone replacement therapies (HRT) have shown to help women maintain much of the bone that is lost during the accelerated bone loss phase and reduce the risk of fractures (102). During this slower phase, cortical and trabecular bone loss is nearly

identical. This secondary phase also matches the bone loss rates observed in males (103).

Bone loss in males from middle age to the end of the lifespan show slow and progressive trabecular and cortical loss. However, since males do not have a menopausal transition to navigate, the overall loss in bone is significantly less than in females. Relative to PMPW, elderly men lose about half as much bone and sustain a third fewer fragility fractures (104). Males experience a two-fold increase in sex hormone binding globulin (SHBG) as they age, which results in a substantial decline in the bioavailability of sex hormones. Specifically, reductions around 47% in estrogens and 64% in testosterone were observed based on data from the Rochester, MN cohort (105). Although testosterone is often considered the primary sex hormone for males, estrogen concentrations have stronger correlations to BMD at several sites in males, suggesting that the bioavailability of estrogen is a more potent factor for skeletal homeostasis (106). Testosterone supplementation studies have tried to characterize the hormone's effects on bone mass; however, it is difficult to elucidate a true mechanism as testosterone can also be aromatized into estrogen (107).

PTH levels also change throughout the aging process and can contribute to the age-related bone loss observed in both sexes. The decreases in serum E2 concentrations that occur during menopause will result in an increase in serum calcium concentrations due to increased bone resorption (108). Increased bone resorption will result in an increase in serum calcium concentrations and cause a compensatory decrease in PTH secretion (108). An opposite PTH response is observed in males and females in the later years of life, resulting in secondary hyperparathyroidism (109). The mechanisms

behind this chronic elevation in PTH in females are twofold; first is reduced active vitamin D, and the second is altered calcium balance due to prolonged E2 deficiency. Active vitamin D synthesis is reduced due to decreased sun exposure and the loss of intestinal vitamin D receptor sensitivity (110). With less active vitamin D, calcium absorption in the small intestine will decrease causing PTH secretion. The second factor is chronic E2 deficiency. Estrogens are needed to maximize the reabsorption of calcium from the kidneys and, without its effects, more calcium is excreted (111). Together with PTH, vitamin D aids to regulate calcium concentrations that are vital to skeletal health (91). Typically, about 200 mg of calcium are removed from the skeleton and replaced each day (112). As previously described, reduced serum calcium concentrations caused by insufficient dietary intake or increased renal excretion will result in bone resorption via PTH. Chronically elevated PTH has been shown to be catabolic to the bone and can increase the risk for low BMD and bone injury (113). Dietary calcium at sufficiently high levels of 1,000 mg/day have been shown to reduce the bone remodeling rate by 10-20% in older adults; however, as the aging process progresses, the vitamin D receptors (VDR) in the intestine become less sensitive, which may contribute to reduced bone mass over time (110). Additionally, the concentrations of 7-dehydrocholesterol, the precursor to vitamin D, is reduced in aging populations, resulting in less vitamin D synthesis from sunlight exposure (114). Adequate vitamin D and calcium levels have been shown to be associated with increased bone mass, strength, muscle mass, gait speed, balance scores and reduced risk of fractures and falls in both elderly men and women (115-117).

Bone health can be greatly affected by inadequate vitamin and mineral consumption, such as vitamin D, calcium, phosphorus, and magnesium (118). Good sources of these vitamins and minerals include fruits and vegetables that are high in phytochemicals. Additionally, fruits and vegetables can act like antioxidants and decrease the aforementioned detriments of ROS on bone (119). Fruit and vegetables also help to maintain a less acidic environment, which can have a positive effect on bone by reducing calcium resorption (120). Protein, on the other hand, has been shown to increase the acid load to bone, which could result in the mobilization of calcium from bone; but in general high protein diets have been associated with increased BMD and decreased fracture risk (120). Both macro- and micro-nutrients are important for skeletal health and are often inadequate in athletes, especially females (121, 122). Low energy availability uncouples bone turnover and suppresses bone formation (123). This caloric deficit may be intentional due to disordered eating patterns and/or excessive exercise. This phenomenon, once thought to only occur in females, termed the female athlete triad, has now been adapted to be called relative energy deficiency in sports (REDS) to expand this concept to all athletes (124).

Drug abuse can also adversely affect bone health; two drugs of common use in the general population and military personnel are alcohol and tobacco. It has been demonstrated that alcohol has a dose-dependent effect on bone with moderated consumption posing beneficial attributes while excessive consumption can lead to decreased BMD (125). Too much alcohol has been shown to increase osteocyte apoptosis, oxidative stress, and Wnt signaling pathway dysfunction (125). A dose-dependent relationship between tobacco use and bone health is well documented;

however, since tobacco causes dysfunction in so many systems throughout the body, a clear mechanism has not yet been characterized (126, 127).

Mechanotransduction

Bone is sensitive to mechanical stimuli; however, certain loading profiles are more osteogenic than others. Over 100 years ago, Roux and Wolff proposed that bone architecture is determined by mathematical laws; then Pauwels, Thompson, Turner, Frost, Hert, Rubin, McLeod, and others continued to further characterize how bone is a dynamic tissue capable of adapting to loads (71, 128, 129). Although many scientists over the past century have added information to the characterization of osteogenic loads, two of the most prolific contributors were Turner and Frost (71, 72, 130, 131).

Turner described three basic rules that a load or stimulus must meet or exceed in order to elicit an anabolic skeletal response (130). First, the load should be a dynamic movement instead of static. Second, short durations of loading are sufficient to induce changes. And lastly, bone cells will adapt to the stimuli over time, thus requiring a progression or novelty of the stimuli. In conjunction with these rules, Frost elaborated on Turner's rules with the introduction of the mechanostat theory. This theory suggests that bone cells have a minimum and maximum threshold of stimuli that will determine the bone response of either conservation of bone mass or the alteration of bone mass by formation or resorption (71). Additionally, four specific aspects of the loading profile may be manipulated to satisfy the aforementioned laws: frequency, magnitude, duration, and rest. The mode to which a load is transmitted to the skeleton will alter these variables and in turn dictate the cellular response.

Arguably, one of the most important controllable factors for bone strength is skeletal muscle capacity. For instance, years after paraplegia or paralysis, bones in the paralyzed lower extremities lose nearly 40% of their strength and mass as compared to the upper body (132). Skeletal muscle has the ability to apply or attenuate stress to the skeleton. The application of stress is most notable during force production at the ends of the long bones. For instance, even during normal locomotion over 2 kg of force generated by the muscles is required to move each kg of body weight (133). In certain regions of the body, bone loading is increased by an internal muscular force as a result of contraction; however, this contraction also decreases the bone loading observed in other regions of the bone (134, 135). Depending on the anatomical locations of the points of insertion and origin, paired with pennation angle, a muscle can provide compression, tension or bending strains to a bone. Additionally, if an external load is applied to the bone from vertical ground reaction forces (vGRF), the compensatory muscle contraction can reduce the compression, shear, torsional, or bending forces applied to the long bones. Individuals, who vary in muscle strength, will be able to produce and redistribute different magnitudes of load (19). This result suggests the same exercise, being performed by two people of unequal muscular strength, may result in unequal osteogenic effects.

To investigate the role of skeletal muscle load attenuation and fatigue, Milgrom and colleagues used a gastrocnemius fatigue model in military recruits (136). Participants completed a 2-km run and 30-km desert march separately, with gastrocnemius fatigue and tibial compression strain rates being measured post-exercise. Results suggested the fatigued state increases bone strains and may be a major factor in

the origins of tibial stress fractures in this population. Additionally, in endurance sports, even early stages of muscle fatigue have been shown to affect the total bone load, specifically the strain rate (137, 138). Not only muscle strength but limb length may be a contributing factor to altered load attenuation. Conflicting studies argue that discrepancies in leg length increase the risk for stress fractures in both military and athletic populations (17, 28, 139-141). It has been hypothesized that the longer the bone, the greater the magnitude of the bending moment caused by both tensile and compressive forces; however, studies show the fractures do not seem to occur preferentially in either the longer or the short leg (8). To mitigate possible injury, manufacturers have turned to a variety of footwear options to reduce the vGRFs the lower body must endure during activity. Six randomized controlled trials have evaluated the effect of custom insoles on the prevention of stress fractures in military populations (142-148). Results suggest the use of the insoles may reduce the number of stress fractures by over 50% (149).

A bone's ability to resist injury is predicated on two properties; structural stiffness and toughness. Structural stiffness is determined by the bone's material properties and structural toughness is determined by the spatial distribution or geometry of the mineralized matrix. These properties in relation to the origin and direction of the applied load will dictate the risk for bone injury (150). Under normal circumstances, microdamage from mechanical loading stimulates bone formation. Unfortunately, with overtraining, the bone is subjected to increased loading with inadequate rest periods, resulting in accelerated and imbalanced remodeling where resorption will become more prevalent than formation leading to decreased structural integrity (4, 151). These

overuse injuries, often also referred to as stress fractures, frequently occur in lower limb bones, especially the tibia, and are highest on the subperiosteal surface (152). In elite military training programs, exercise conditions are relatively uniform between recruits; however, fracture rates are not. Females and smaller recruits tend to report more stress fractures, which may suggest a difference in either biomechanical or bone geometry that results in the increased risk (19). Studies on Israeli Army recruits showed that participants with fractures had more narrow tibiae and smaller tibial mediolateral cross-sectional moments of inertia (CSMI) (15, 153). Beck et al. (19), also found that, independent of body size, those who suffered stress fractures were more likely to have smaller section moduli in the femur and tibia.

Bone Status Assessment Techniques

The first documented attempts at quantifying aBMD were in the late 1800's from dental radiographs (154). The importance of this field quickly grew and by the late 1980's Hologic released their DXA technology. DXA uses the basic principle of X-ray attenuation to measure tissues. In general, X-rays high and low photon energies are passed through the participant and the attenuation of those two energies is measured by a detector. Tissue thickness, density, and composition will alter the attenuation profile of each energy beam, which is then used to characterize that tissue in a 2D planar image (155). DXA has now become the gold standard for measuring aBMD because of excellent accuracy and precision values paired with low radiation exposures for participants (156, 157). Today, DXA machines have a wide range of capabilities that include total body and site-specific tissue composition quantification, such as bone mineral content and density, fat mass, bone free lean body mass, hip structural analysis,

abdominal aortic calcification and trabecular bone score measurements (158-160). These measures can be further used to diagnose osteoporosis, fracture risk, and monitor changes in body composition and aBMD over time (157, 161). For aBMD measures, T-Scores and Z-Scores are also reported on the DXA output, which can be used to define low bone mass, osteopenia, and osteoporosis. For individuals under the age of 50 years, a Z-Score will report the number of standard deviations a participant's aBMD is above or below what is normally expected for an age, sex, body mass, and ethnicity matched participant. Scores that are above -2.0 are considered normal, while scores that are below -2.0 are considered low bone mass (65). For individuals over the age of 50 years, a T-Score reports the number standard deviations a participant's aBMD is above or below what is normally expected for a Caucasian, female aged between 20-29 years old (65). Scores that are -1.0 and above are considered normal, while scores that range from -1.1 to -2.4 are defined as having osteopenia, and $\leq$-2.5 is defined as having osteoporosis (65). Bone tissue mineralization and distribution are independent predictors of bone strength, and the combination of both of these properties improves the estimation of bone strength and fracture risk (162). As mentioned previously, DXA can be used to provide the extent of mineralization; however, the distribution or geometry of this tissue is better captured using volumetric measures such as pQCT (163, 164). Much like DXA, pQCT technologies had many precursors that began around the late 1960's and early 1970's. This imaging technique quickly began to evolve and by the early 1990's, QCT scanners were being used to image the lumbar spine and hips and a peripheral QCT had been developed to assess the appendicular skeleton (165, 166). The pQCT uses the same general concept of X-ray beam

attenuation as the DXA. However, the source and the detector can simultaneously rotate around a 180° axis to generate a 3D, volumetric measure instead of a 2D planar measure. pQCT allows researchers to scan nearly any segment of the appendicular skeleton and yield bone quality and muscle cross-sectional area results without exposing the participant to large radiation doses. Measures of vBMD, content, area, circumferences and a series of bone strength indices provide information about skeletal geometry. The type of bone is very important for understanding fracture risk, as cortical and trabecular bone play different roles in bone strength and quality and can be differentiated by pQCT (167). DXA and pQCT are valuable skeletal assessment tools separately, as they provide different types of information about the bone tissue; however, when used in conjunction, they provide a more complete understanding of tissue density, content, geometry and quality.

Bone Turnover Markers (BTM) are circulating biomarkers that allow researchers to make inferences about the real-time skeletal response to stimuli such as loading or unloading (20). BTM have been identified for both resorption and formation and can be assayed from small samples of blood or urine. For example, biomarkers such as serum procollagen type I N propeptide (PINP), bone alkaline phosphatase (bone ALP), osteocalcin (OC) are commonly used as markers for bone formation, while N or C-terminal telopeptide of type I collage (NTX-I or CTX-I), tartrate-resistant acid phosphatase (TRAP family) are commonly used to describe bone resorption (21). Specifically, PINP and CTX-I are bone turnover markers recommended by the International Osteoporosis Foundation (168).

In general, the three previously mentioned bone formation markers are expressed due to osteoblast activity. OC is a binding protein exclusively synthesized by osteoblasts that aids in the early organization of the matrix as it has specific binding sites for calcium (169). Unfortunately, OC is not protected from rapid degradation in serum, so assays have been developed to quantify both the OC fragments and fully intact OC molecules (20). Bone-ALP is an enzyme that aids osteoid formation and mineralization (20). In serum, nearly half of all alkaline phosphatase is from the liver, with the other half coming from bone. Assays used in skeletal research only quantify the bone-derived isoforms. PINP, the last bone formation marker, is in a class of peptides that is specific to proliferating osteoblasts (22). It has very low individual variability and is not subject to large changes due to the circadian rhythm (22).

Bone resorption markers are associated with a variety of cells. Tartrate-resistant acid phosphatase has six isoenzymes that are found in many tissues; however, TRAP5b is specific to bone osteoclasts. TRAP5b is secreted from the osteoclasts' ruffled edge during migration from one resorption pit to another and is used as a marker of mature osteoclast number and bone resorption activity (25, 170). When osteoclasts resorb bone, a variety of proteins are released; however, 90% of these fragments are type I collagen. NTX-I and CTX-I refer to a terminal collagen crosslink that is either on the carboxyl or amino end of the type I collagen. Both NTX-I and CTX-I provide evidence for osteoclast activity and bone resorption; however, CTX-I is more commonly used as it has more stable resting values as compared to NTX-I and has now consistently been strongly correlated with changes in BMD (24).

BTM have been shown to be strong predictors of stress fracture risk in clinical populations (26, 27). However, the evidence for BTM correlation to stress fracture is inconsistent within military groups. Prospective studies have found that BTM were strong predictors of injury status (30), while other studies demonstrated no relationship at all in military (3, 17, 31). New biomarkers, such as PTH and sclerostin need to be investigated as potential correlates of injury in military populations.

Parathyroid hormone (PTH) is responsible for the maintenance of serum calcium concentrations needed for normal bone health, muscle function, and many other processes. PTH has multiple mechanisms by which it can mobilize calcium: promoting bone resorption, reducing urinary calcium excretion, while simultaneously increasing phosphate excretion, and by increasing calcium absorption from the small intestines indirectly, and by activating Vitamin D. In general, PTH and calcium have an inverse relationship; short term increases in calcium levels will reduce the release of stored PTH from secretory vesicles and trigger the degradation of PTH into fragments (171). If serum calcium levels are consistently low, PTH levels will remain elevated. Chronically depressed or elevated PTH concentrations, as seen with hypo- and hyperthyroidism, can be detrimental to skeletal health; however, intermittent PTH administration has been shown to have anabolic effects on bone (172, 173).

PTH responses to acute and chronic exercise are inconsistent. Scott et al. (174) demonstrated that PTH increased with a single bout of treadmill running in healthy males. Furthermore, they speculated that the increases in PTH concentrations after participants reached a workload of 75% VO_2 max is due to decreases in serum calcium concentrations (174). Sherk et al. (175) investigated the PTH response to a single bout

of cycling, with and without calcium supplementation, to test this idea. They hypothesized that a 1,000 mg calcium chewable supplement taken 30 min prior to the cycling bout would protect PTH levels; however, there was no significant difference in post-exercise PTH responses between those subjects who received the supplement and those who received the placebo (175). However, an acute bout of vigorous walking in postmenopausal women revealed that calcium supplementation before and during the bout did protect PTH responses (176). PTH responses to chronic exercise have also been inconsistent. Lester et al. (6) investigated exercise mode over eight weeks in women who reported fewer than two days per week of PA. Regardless of exercise mode, all groups demonstrated an increase in PTH from pre- to mid-training; however, these serum PTH concentrations returned back to pre-training levels by the end of the eight week training intervention (6). High serum PTH levels have been found to be associated with stress fracture in military recruits (17, 34); however, contrary findings have been observed in athletic and military populations (31, 35, 177-179).

The Wnt/β-catenin signaling pathway has clearly been characterized as an osteogenic process that is tightly regulated. Wnt proteins are a family of secreted proteins that can bind to a 7-transmembrane frizzled receptor and low-density lipoprotein receptor protein 5 and 6 (LRP5/6). Once this binding occurs, signals are generated, in part due to Disheveled, Axin, and Frat-1, that will inhibit GSK3's ability to destabilize β-catenin. This stabilized β-catenin will translocate to the nucleus, where it interacts with T cell factor lymphoid enhancer binding factor (TCF/LEF) to increase transcription of proteins and lead to downstream osteogenesis (180). Wnt/β-catenin

signaling can be inhibited by an interaction with the LRP5/6 receptor and sclerostin; resulting in decreased osteoblast differentiation (37).

Mechanical loading and hormones such as PTH, calcitonin and glucorticoids can reduce sclerostin production (181). Animal research has demonstrated that deletion of the SOST gene or sclerostin KO models have significantly greater bone formation and strength as compared to wild type mice (182), and mechanical loading will reduce the amount of sclerostin produced by the osteocytes (39). In humans, sclerostin responses are less consistent. Many cross-sectional studies in adolescent and adult athletic populations have shown sclerostin to be increased as compared to less active controls or increased after an acute exercise bout. Falk et al. (38) compared sclerostin responses to exercise in boys and men and found that boys had greater resting values of the protein, but men had a significantly greater increase in sclerostin post-exercise (38). Women who reported low levels of PA, slowly jogged on a treadmill for 45 minutes, which resulted in a nearly 45% increase in serum sclerostin levels (183). Adolescents who were involved in athletics had higher sclerostin concentrations than matched non-athletes (184), rugby and endurance sport athletes (185) and soccer players (181) all had greater sclerostin as compared to matched controls. However, most of these cross-sectional studies did not account for plasma volume shifts during the acute exercise bout, which could result in the overestimation of sclerostin production. Many of these studies only collected pre/post exercise bout blood samples, which might only characterize the brief catabolic state of the bone immediately post-exercise (176).

In postmenopausal women followed over a five year period, sclerostin was a significant positive predictor of fracture (43). Unfortunately, only a few studies have

assessed the effects of PA on sclerostin in humans over time. Sclerostin was shown to increase during a nine day stage race in nine professional cyclists (186). Nine participants completed a 246-km ultra-distance race and had blood serum samples collected before, during, and after participation. The average race time was just over 34 hours for the nine participants. Serum sclerostin levels were not different from pre- to post-blood draws but were significantly reduced by the third day after the race (41). Both of these studies represent such intensive bouts of PA, over short durations of time, which make the generalizability of the results difficult. The only longitudinal intervention assessing sclerostin concentrations and PA was a 12-month exercise intervention in males with low spine or hip aBMD. Participants either engaged in resistance training or high-intensity jump training, two or three times per week, respectively. Both training programs were periodized and progressive. Serum sclerostin levels were reduced while aBMD increased in both groups (42).

Exercise Interventions in Military Populations

As previously mentioned, both drug and exercise interventions have been prescribed to cohorts at risk for bone injury, including military personnel. In 2004, Milgrom et al. (187) hypothesized that acute suppression of bone turnover using bisphosphonates would decrease the incidence rate of stress fractures in 324 new male infantry recruits. Participants received either a placebo or bisphosphonate risedronate during the initial stages of training. A weekly maintenance dose was also administered for the duration of the study. The researchers found no significant reduction in fracture risk over the training period (187).

Many risk factors for bone injury and fracture can be positively influenced by regular physical activity, so it seems more logical to rely on an intervention of this type to maximize skeletal benefits. However, in certain populations, improper prescriptions of exercise and rest periods can result in additional bone injuries. The military has completed a variety of studies on the most efficient types of exercise regimens to increase strength, power, endurance, and operation specific skills while simultaneously reducing the risk for musculoskeletal injuries in their tactical athletes. Throughout the 1990s, the Defense Women's Health Research Program began investigating different training protocols specifically to facilitate a better transition for female military recruits. The first of three studies was conducted by Knapik, et al. (188). Thirteen female recruits participated in a 14-week training program that consisted of three days/week of resistance training (RT) and two days/week cardiorespiratory training (AT). Testing variables included strength, body composition, and manual material handling (MMH) lifting tasks. Knapik reported 9% decreases in fat mass, 6% increases in lean mass and a 16-19% increase in the MMH tasks. A second study conducted by Harman et al. (189) employed a more extensive functional testing program which included higher training volume, and a periodized model of resistance training including load carriage, plyometrics, interval training and more mission specific tasks, 5 days/week (189). Participants increased their 1 repetition maximum (1RM) by 30-47%, repetitive lifting capacity by 18-32%, load carriage ability by 24%, aerobic capacity by 14%, and decreased reported musculoskeletal injury. The third study by Kraemer et al. (190) followed 83 college-aged women and 100 untrained male controls over a three year period where six different, six month training programs were implemented. All

programs were conducted 3 days/week. Participants either engaged in AT or RT only, or the combination of the two. These combination protocols were divided by power and hypertrophy repetition ranges and upper and lower body. Outcome measures included a large battery of neuromuscular tests, muscle cross-sectional area via MRI, functional tests of a 1RM box lift and repetitive box lifting tasks, plus a 2-mile 75-lb load carriage walk. The primary findings revealed that programs using power repetition ranges, resulted in the greatest attenuation of the sex physical performance gap. This study might also provide evidence that untrained women need at least six months, and maybe more, as a training plateau was not observed for power and strength (190). Subsequently, the Army began updating their training protocols in the early 2000s (50).

In 2010, the U.S. Army launched the Soldier Athlete Initiative, charged with improving physical performance among entry level recruits by changing training tactics from traditional push-ups, sit-ups and endurance runs to more strength, power, agility, and core training in addition to updated nutrition and injury prevention strategies (7, 191). The National Strength and Conditioning Association's Second Blue Ribbon Panel of Military Physical Readiness stated strength and power are the two most important aspects of successful completion of military tasks while reducing injury rates (192). Part of the training doctrine changes implemented in the early 2000s included the reduction of long distance running and the inclusion of more high intensity interval training (HIIT). HIIT has been shown to have positive biochemical effects in as short as two weeks (177, 193). Burgomaster et al., (193) demonstrated that over six sessions in two weeks (only 15 minutes of total exercise time) there were significant increases in citrate synthase (38%), resting muscle glycogen (26%) and cycle endurance capacity

(100%). Later work also highlighted a significant increase in the maximal activity of cytochrome *c* oxidase (COX) and COX II, suggesting greater electron transport chain capacity (194). Gibala et al.'s research protocol required only 2.5 hours of interval training as compared to the endurance training group that worked 10.5 hours (194). HIIT provides great potential for positive mitochondrial adaptations in shorter periods of time as compared to traditional AT training. One concern is that concurrent AT more than four times per week above 80% VO_2 max has been shown to override positive neuromuscular adaptations to strength and power gains from RT (195). This has been termed the interference effect. Hendrickson et al. (196) demonstrated a minor interference effect in female military recruits; however, the concurrent AT and RT still resulted in an increase in all occupational task scores as compared to AT or RT alone.

Summary

Uncoupled and dysfunctional signaling of bone cells may result in low BMD, which can increase the risk of fracture or bone injury. Many factors are implicated in this process; however, only a few are controllable. Dietary, pharmaceutical, and exercise interventions have demonstrated attenuation of bone loss in a variety of populations, including military cohorts. The occupational demands of military personnel are unusual and can result in overuse musculoskeletal injuries; however, the exact relationships between aerobic fitness, strength, power, and skeletal metabolism are not well characterized. New assessments are needed to better understand what factors put military personnel at risk for fractures.

Chapter 3: Methodology

The purpose of this study was to assess the effects of an eight week MTIP, on changes in PTH and sclerostin serum concentrations, and aBMD of the total body, dual femur, and lumbar spine, and bone geometry of the non-dominant tibia in healthy, college-aged, Marine Corps. and Naval ROTC members compared to a matched control group. Additionally, the relationship between these biomarkers and parameters of bone health were examined. Lastly, body composition, upper and lower body muscular strength and power measures and aerobic fitness were compared between groups and the correlation between biomarkers and bone parameters was determined.

Participants

In total, 42 participants (ROTC members, n=20; controls, n=22), were enrolled in the study; however, due to attrition only 36 participants were included in the analysis (ROTC members, n=18; controls, n=18). Eight females (ROTC members, n=4; controls, n=4); 4 of which reported using oral contraceptives while 1 female reported using a Nexpalnon implant. 31 participants (ROTC members, n=16; controls, n=15) who completed pilot testing during the fall semester (IRB#8338) were again recruited for participation in the current study. Other OU ROTC participants were recruited after the OU Naval ROTC commanding officer, Captain Lyle Hall, granted permission (Appendix B). Captain Hall and the OU Naval ROTC program oversee both USMC and Naval ROTC students and organized group meetings for recruitment opportunities. All control participants were recruited from the University of Oklahoma using word of mouth and fliers. Participants were informed of the risks and benefits before providing written consent prior to testing. All procedures were approved by the Institutional

Review Board at the University of Oklahoma Health Sciences Center (OUHSC IRB # 8600).

Experimental Group Inclusion Factors

1. Participants were males and females between 18-30 years old.
2. Participants were active members of either the Marine Corps or Naval OU ROTC programs.
3. Participants were healthy and free of any diseases or disorders known to impair skeletal health or limit their ability to perform vigorous exercise such as osteoporosis, cardiovascular disease, or uncontrolled hypertension.
4. Women were screened for having regular menstrual cycles, defined as not having more than three consecutive months without a period. Women who reported using hormonal contraceptives were not excluded.
5. Body weight was less than 300 lbs (136.3 kg), and height was less than 76 in (1.92 m) which are the limits of the DXA machine.

Experimental Group Exclusion Factors

1. Women who were pregnant or planning on becoming pregnant.
2. Individuals who had a history of taking medications known to affect BMD, such as glucocorticoids, bisphosphonates, and calcitonin.
3. Individuals who had metal implants in the spine, hips or legs.
4. Individual who were current smokers or who had smoked within six months.

Control Group Inclusion Factors

1. Participants were sex, age (±2yrs), and body mass (±2.3kg) matched to a ROTC member participant.

2. Participants engaged in physically activity at least three times per week.
3. Participants were healthy and free of any diseases or disorders known to impair skeletal health or limit their ability to perform vigorous exercise such as osteoporosis, cardiovascular disease, or uncontrolled hypertension.
4. Women had regular menstrual cycles, defined as not having more than three consecutive months without a period.
5. Body weight was less than 300 lbs (136.3 kg), and height was less than 76 in (1.92 m) which are the limits of the DXA machine.

Control Group Exclusion Factors

1. Women who were pregnant or planning on becoming pregnant.
2. Individuals who had a history of taking medications known to affect BMD, such as glucocorticoids, bisphosphonates, and calcitonin.
3. Individuals who had metal implants in the spine, hips or legs.

Research Design

This was a mixed factorial research design with one within-subjects variable (time) and one between subjects variable (group). Pre and post an eight week MTIP blood draws were used to determine changes in PTH and sclerostin serum concentrations, and aBMD of the total body, dual femur, and lumbar spine, and bone geometry of the non-dominant tibia in healthy, college aged, Marine Corps and Naval ROTC members as compared to a matched control group. Additionally, the relationship between these biomarkers and parameters of bone health were examined. Lastly, body composition, upper and lower body muscular strength and power measures and aerobic fitness were compared between groups and the correlation to biomarkers was

determined. The eight week MTIP during the Spring of 2018, had three time points of data collection in January (pre), March (mid) and April (post) as shown in Figure 1. All visits took place in the Bone Density Research Laboratory and the Neuromuscular Performance Laboratory.

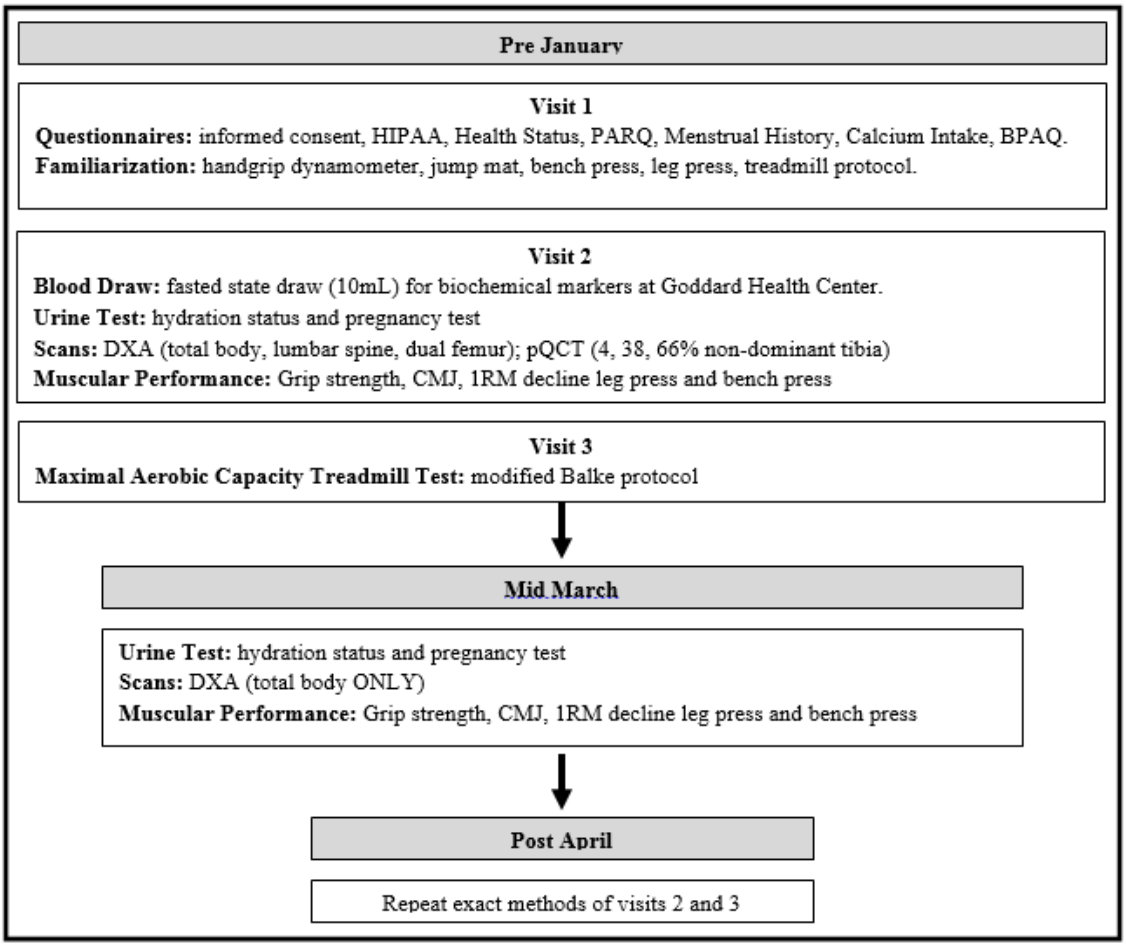


Figure 1. Overview of the Recruitment Process and Research Design.

For the first visit, participants completed the consent process and filled out a series of questionnaires including a Health Status Questionnaire, a Physical Activity Readiness Questionnaire (PARQ), a Menstrual History Questionnaire, a Calcium Intake Food Frequency Form, training logs and a Bone Specific Physical Activity Questionnaire (BPAQ). Participants also became familiar with the methods required for

using a hand grip dynamometer, jump performance testing, bench press, leg press, and the maximal graded exercise treadmill test. The individuals who qualified for the study based on pre-screening were scheduled for the second visit. The second visit consisted of a blood draw, urine sample, bone scans, and physical performance testing. The serum was used to quantify the biomarkers, PTH and sclerostin. The urine sample was used to check hydration values and female pregnancy status. Four DXA scans (total body, lumbar spine, dual proximal femur) and three pQCT scans (4%, 38%, 66% length of the non-dominant tibia) followed the blood draw. Also, body composition variables (total and regional percent body fat (%BF), FM, BFLBM) were obtained from the total body DXA scan. Upper body strength testing involved grip strength and a 1 repetition maximum (1RM) bench press. Lower body strength testing included a jump performance test and a 1RM leg press. Lastly, the third visit consisted of a modified Balke graded exercise treadmill protocol that was used to measure peak VO_2 (mL/kg/min).

Questionnaires

All participants completed several forms of paperwork and questionnaires during the first visit. The following items provided researchers screening information and also important classification data.

1. Informed Consent and HIPAA - these forms were used to ensure the participant had a complete understanding of the study procedures including potential risks and benefits.

2. Health Status Questionnaire - is an in-house questionnaire used to provide additional screening information to ensure the participant was qualified for the study and it was safe for them to participate.
3. Training Questionnaire - this form was used to describe auxiliary forms of PA in which the participants were engaging in outside of the intervention and any musculoskeletal injuries they may have sustained during this time, such as shin splints or stress fractures.
4. Bone-Specific Physical Activity Questionnaire (BPAQ) – was used to quantify bone loading activities that participants were engaged in. Total, past, and current BPAQ scores were calculated (197).
5. Physical Activity Readiness Questionnaire (PARQ) – was used to see if participants could begin a physical activity program, by screening for diseases.
6. Calcium Intake - was assessed before the MTIP began, using a calcium-rich foods frequency questionnaire (198).
7. Menstrual History - an in-house menstrual history questionnaire was used to describe female participant menstrual cycle characteristics over the past 12 months and contraceptive use, age at menarche, symptoms of menstrual cycle and hormonal disturbances.

Anthropometric Measures

Body mass and height were measured using a Tanita BWB-800 digital scale (Tanita Corp. of America Inc., Arlington Heights, IL) and a wall-mounted stadiometer.

Urine Testing

For measuring hydration and pregnancy status in females, the participants provided a small urine sample. To measure hydration, a VEE GEE-CLX-1 refractometer (VEE GEE Scientific Inc, Kirkland, WA) was used to measure urine specific gravity. All participants were reminded to come to the lab hydrated and ready to provide a urine sample. Samples had to be within 1.004-1.029 in order to undergo a DXA scan. If participants were dehydrated, they were given water and retested after 30 minutes, if participants were over-hydrated they were asked to reschedule the visit. Female participant urine samples were also used to test for pregnancy using a pregnancy strip (SA Scientific, San Antonio, TX). The urine sample was allowed to reach room temperature and the pregnancy strip was dipped into the urine for 15 seconds and then left to rest for four minutes, after which time the strip was read.

Dual Energy X-ray Absorptiometry

DXA (GE Lunar Prodigy, enCORE software, version 13.31.016, GE Healthcare, Madison, WI) was used to measure whole body composition and aBMD. Measures of total FM (g), %BF, BFLBM (g), and bone mineral content (BMC) (g) were obtained from the whole body scan. aBMD is measured using specific scans of the total body, lumbar spine (L1-L4), and dual proximal femur (total hip, femoral neck, trochanter). For the total body scan, participants were asked to lie on the DXA table in the supine position, centered within the scan field. The hands were placed on the sides of the legs, in the prone position, while the legs were straight and strapped together. Participants remained centered and placed their legs on a foam block so the lumbar spine was completely flat for the lumbar spine scan. Lastly, for both proximal femur scans, the

feet were strapped to an angled brace to create internal rotation of the femur. In the Bone Density Research Laboratory, the root mean square coefficient of variation (RMS CV %) for body composition variables for Total FM, BFLBM and %BF are 2.74%, 1.39%, and 2.5%, respectively. The *in vivo* RMS CV % for the aBMD of total body is 0.6%, L1-L4 is 0.9% and 0.4-0.8% for the proximal femur sites. The same trained technician conducted all scans.

Peripheral Quantitative Computed Tomography

A pQCT scanner (XCT 3000, Software v.6.00, Stratec Medizintechnik GmbH, Pforzheim, Germany) was used to measure tibia bone geometry characteristics and muscle cross-sectional area (mCSA). Tibia length of the non-dominant limb was measured from the medial malleolus to the tibial plateau. Leg dominance was defined as the participant's self-reported preferred kicking leg. Participants were seated with their leg supported horizontally and centered in the gantry. Tibia scans were obtained at 4%, 38%, and 66% of tibia length proximal to the reference line. A voxel size of 0.4 mm was used for all sites at the scout view speed of 40 mm/sec and CT speed of 20 mm/sec. At the distal tibia (4%), contour mode 3 at 169 mg/cm^3 and peel mode 4 at 650 mg/cm^3 with a 10% peel were used to determine total vBMD (mg/cm^3), total bone area (mm^2), trabecular vBMD (mg/cm^3), and trabecular area (mm^2). For the 38% and 66% tibia sites, cort mode 2 at 710 mg/cm^3 was used to define total vBMD (mg/cm^3), total bone area (mm^2), cortical density (mg/cm^3), cortical area (mm^2), and cortical thickness (mm), while cort mode 2 at 480 mg/cm^3 was used to obtain torsional strength for strength-strain index (SSI) (mm^3). In the Bone Density Research Laboratory, the RMS CV% for

the pQCT bone measurements ranges from 0.31-1.21% for all sites. The same trained technician who performed all DXA scans also conducted all pQCT scans and analysis.

Muscular Strength and Power Measures

All participants completed two upper and two lower body specific tests of strength and power. Lower body muscle power was assessed by a maximum counter movement jump (CMJ). Using a validated jump mat (Just Jump, Probotic, AL) with a Tendo FiTRODYNE power and speed analyzer (Tendo Sports Machines, Trencin, Slovakia), participants jump power (w), velocity (m/s), time in the air (s) and vertical jump (cm) was recorded. Participants were instructed to squat down to a self-selected depth and then use a forceful arm swing to jump as high as possible. Each participant jumped three times with a one-minute rest between trials. The Bone Density Research Laboratory Intraclass Correlations (ICC) values for jump power, velocity, air time, jump height range between 0.80-0.98. Additionally, lower body strength was determined using a decline leg press machine (Body Solid, Forest Park, IL). An eight trial 1RM protocol was used, per National Strength and Conditioning Association recommendations (199). The same 1RM protocol was used for the upper body strength assessment using bench press (Cybex, Medway, MA). The Neuromuscular Laboratory ICCs for leg press and bench press 1RM testing are 0.997 and 0.999, respectively (200). Lastly, grip strength was measured using the Jamar handgrip dynamometer (Patterson Medical, IL) (201). The ICC for this handgrip dynamometer in the Bone Density Research Laboratory is 0.874.

Graded Exercise Treadmill Testing

Maximal aerobic capacity was measured using a modified Balke treadmill protocol with open-circuit spirometry (ParvoMedics; Sandy, UT). Participants warmed up to determine the jogging speed that elicited a heart rate equivalent to 75% of age-predicted maximal heart rate. This speed was maintained or increased while the treadmill grade was increased by 2% every 2 minutes. Heart rate was monitored continuously using a coded transmitter worn around the chest (Polar T31, Bethpage, NY). Prior to the end of each stage, participants were asked their Rating of Perceived Exertion (RPE) (202). VO_2 peak was calculated as the average of the two highest consecutive 30-second VO_2 measurements (176).

Four criteria were used to identify maximal oxygen uptake; a plateau in oxygen consumption despite an increased workload, RER values over 1.10, RPE values 18 or over, and maximal HR within 10 bpm of the age predicted max HR. Average RER was 1.14, average max heart rate was 196bpm, and average RPE was 18.7. After visual inspection of VO_2 kinetics, 56 of the 64 exercise tests demonstrated a clear plateau in oxygen consumption. Of the eight tests that were not clear six tests reached all of the other criteria for max.

Exercise Intervention

OU ROTC participants completed the same biweekly, eight week structured training programs within each branch. All USMC OU ROTC members completed the same frequency, time, and type of exercises; however, the intensity for each exercise could have been different for each participant based on ability and effort. Navy OU ROTC members also completed the same frequency, time, and type of exercises;

however, these were not always the same as the USMC OU ROTC members. The Spring 2018 MTIP began January 29th and ended April 5th. Exercises conducted by both branches could be categorized as body weight work, resistance training, or endurance training. For instance, in one day OU ROTC members might have completed 100 push-ups, 100 sit-ups (body weight work), followed by a 3-mile run (endurance), 3x5 leg press (resistance training). Each exercise incorporated different aspects of all three types of exercises. Figure 2 shows an example of a USMC OU ROTC circuit that includes both body weight work and resistance training components.

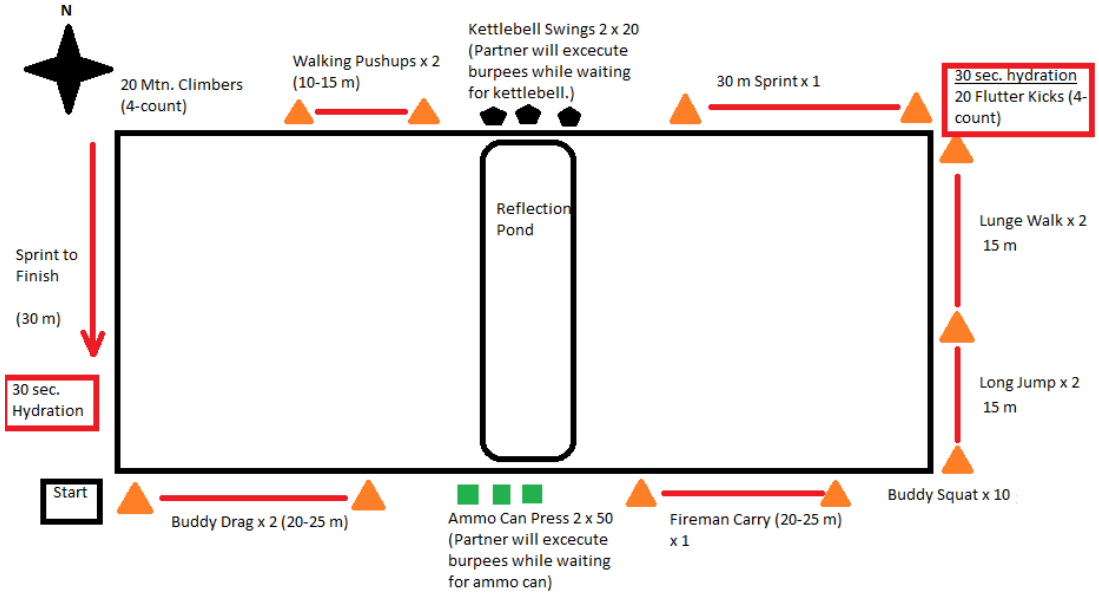


Figure 2. USMC OU ROTC Sample Circuit Workout.

Blood Sampling and Biomarker Assays

Participants were instructed to refrain from PA 24 hours prior, and be at least eight hours fasted for all blood draws. Blood samples (approximately 10mL) were collected via venipuncture by a certified phlebotomist at Goddard Health Center or in the Bone Density Research Laboratory in the morning (8:00-9:00am). Each sample was allowed to clot, then centrifuged to separate the serum from the red blood cells. The

serum was aliquoted into microtubes, labeled, and stored in a -84°C freezer located in the Bone Density Research Laboratory until assays were performed. Prior to each assay, the frozen serum samples and all kit components were allowed to reach room temperature. All samples were assayed in duplicate. The immunoassay kit used for the quantification of PTH was from DRG International Inc., Springfield, NJ. (Cat# EIA3645). Intra-assay CV% ranged from 0.3- 7.7% and inter-assay CV% was 8.0% for the low control and 8.5% for the high control. The immunoassay kits from TECO medical Quidel Corp., Santa Clara, CA and Sissach, Switzerland (Cat# TE1023-HS) were used to measure sclerostin serum concentration levels. Intra-assay CV% ranged from 0.2-9.4% and inter-assay CV% was 20.1% for the low control and 4.7% for the high control. Instructions for each assay kit (Appendix F) were followed as were all standard precautions for Biosafety level 2.

Statistical Analysis

All statistical procedures were performed using IBM SPSS (v23, Armonk, New York), and significance was set at $p \leq 0.05$. Data was tested for normality using the Kolmogorov-Smirnov test and reported as means ± standard deviation (SD) in tables and means ± standard error (SE) in figures. A Mann-Whitney U test was used to analyze baseline differences in calcium intake between groups while Friedman tests were used to detect group and time differences in left hand grip strength as these two variables were not normally distributed. For normally distributed variables, group (ROTC and control) differences in physical characteristics were examined using independent t-tests. No significant differences for age, body mass, or height were found so no covariates were used in subsequent analyses. For variables only measured at the

beginning and end of the intervention period, two-way repeated measures ANOVA was used to determine group (ROTC, control) and time (pre, post) main effects and group × time interactions. If there was a significant interaction effect, the model was decomposed using paired t-tests for pre and post variables within each group. These variables included both biomarkers, lumbar spine and dual femur DXA scans, all pQCT measures, and aerobic capacity variables. For variables which also included a midway testing period, two-way repeated measures ANOVA was used to determine group (ROTC, control) and time (pre, mid, post) main effects and group × time interactions. If there was a significant interaction effect, the model was decomposed and separate one-way repeated measures ANOVAs with Bonferroni post-hoc procedures were performed separately for each group. These variables included all measures from total body DXA scans, and the muscular strength and power measures of grip strength, jump power, bench press, and leg press. Additionally, percent changes were calculated for all dependent variables and were analyzed using independent t-tests with a Bonferonni correction, the equation used was [(post-pre)/post]*100. Pearson Product Moment correlation coefficients (r) were used to determine relationships between biomarkers, bone variables, body composition, and physical performance measures. Lastly, multiple linear regression using the stepwise method was used to identify predictors of total aBMD and bone strength indices.

Chapter 4: Results and Discussion

The purpose of this study was to determine the effects of an eight week military training intervention on PTH and sclerostin serum concentrations, and aBMD of the total body, dual femur, and lumbar spine, and bone geometry of the tibia, in healthy, college-aged USMC and Naval Reserve Officers' Training Corps (ROTC) members compared to a matched control group. A secondary purpose was to determine the relationship between PTH and sclerostin and bone variables. Lastly, group differences in body composition, muscular strength and power measures, and aerobic fitness and their relationships to PTH and sclerostin were examined.

Participant Characteristics

A total of 42 participants (ROTC n=20, Controls n=22) were enrolled in the study. Three control participants were excluded prior to testing due to voluntary termination (n=1), injury (n=1), and an inability to maintain a matching body mass (n=1). Another control participant became injured between the mid and post testing periods and was removed. One ROTC member was excluded prior to testing due to severe illness and another due to voluntary termination after the mid testing period. In total, 36 participants aged between 18-29 years (ROTC n=18, controls n=18) were included in the final analysis. Of the eight females enrolled, (ROTC members, n=4; controls, n=4); 4 reported using oral contraceptives while 1 female reported using a Nexpalnon implant. All matching criteria were maintained for the final 36 participants with no significant changes in height or body mass occurring over time for either group. Hand and foot dominance was determined by asking the participant which hand they threw a ball with, and which foot they kicked a ball with. Four participants reported

being left handed while only two reported being left footed. Participants were not recruited or matched according to ethnicity though the vast majority of participants self identified as Caucasian (n=32); other ethnicities represented were Hispanic (n=2), Black (n=1), and Other (n=1). One control participant reported sustaining a stress fracture due to activity unrelated to the study. No ROTC participants reported any signs or symptoms of any bone injuries throughout the eight week intervention period.

Baseline participant characteristics are found in Table 1. No significant differences existed between groups for age, height, body mass, calcium intake or past, current, and total BPAQ scores (all $p \geq 0.058$). Additionally, no significant group differences were found for the total number of days per week of reported physical activity (PA), resistance training (RT), or endurance training (ET), this information was gathered from the Auxiliary Training Questionnaire (Appendix E). Although not shown in Table 1, body mass index (BMI) ranged from 18.9-26.8 kg/m² with only 11 participants (ROTC n=6; controls n=5) in the overweight category. Calcium intake group means were above the recommended 1000 mg/day (203).

Table 1. Baseline Participant Characteristics (means ± SD).

	ROTC (n=18)	**Controls (n=18)**
Age (years)	20.4 ± 2.4	21.2 ± 1.8
Height (cm)	175.7 ± 9.1	177.5 ± 6.7
Body Mass (kg)	73.4 ± 10.9	74.5 ± 10.7
Calcium Intake (mg/day)	1165 ± 571	1072 ± 725
BPAQ- Past	46.9 ± 27.8	53.2 ± 36.1
BPAQ- Current	6.2 ± 4.2	6.8 ± 5.5
BPAQ- Total	26.6 ± 14.6	29.9 ± 19.0
Days/Week PA	4.9 ± 1.4	4.9 ± 1.0
Days/Week RT	4.1 ± 1.6	3.5 ± 1.8
Days/Week ET	3.5 ± 1.7	2.4 ± 1.8

BPAQ: Bone Physical Activity Questionnaire RT: Resistance Training
PA: Physical Activity ET: Endurance Training

Dual Energy X-ray Absorptiometry Measures

DXA was used to assess changes in aBMD and body composition for the total body and site specific areas. Table 2 shows information regarding the three total body scans that were completed at the pre, mid, and post testing periods. No significant group × time interactions, or main effects for time or group were found for total body aBMD, BMC, or fat mass (all $p \geq 0.083$). Significant time effects were found, total BF% decreased while total body BFLBM increased from pre to mid points (both $p \leq 0.050$). No significant group effects were found for total body aBMD, BMC, BF%, fat mass, or BFLBM (all $p \geq 0.165$). All participants had normal aBMD values according to their Z-Scores per International Society for Clinical Densitometry guidelines (204).

Table 2. Total Body aBMD and Body Composition Over Time (means ± SD).

	Time	**ROTC (n=18)**	**Controls (n=18)**
Total Body aBMD (g/cm^2)	Pre	1.329 ± 0.112	1.348 ± 0.123
	Mid	1.326 ± 0.115	1.338 ± 0.119
	Post	1.314 ± 0.131	1.343 ± 0.116
Total Body BMC (g)	Pre	3023.26 ± 521.22	3020.36 ± 500.37
	Mid	3028.78 ± 513.70	3022.60 ± 505.41
	Post	3040.81 ± 519.32	3019.30 ± 492.58
Total Body % Fat	Pre	20.8 ± 5.5	21.8 ± 6.6
	Mid *	20.3 ± 5.7	21.2 ± 6.2
	Post	20.4 ± 5.8	21.3 ± 6.2
Total Body Fat Mass (kg)	Pre	15.2 ± 4.2	16.1 ± 4.2
	Mid	14.8 ± 4.1	15.6 ± 4.1
	Post	14.9 ± 4.3	15.7 ± 3.9
Total Body BFLBM (kg)	Pre	55.3 ± 9.7	56.5 ± 11.5
	Mid *	55.9 ± 10.1	57.0 ± 11.7
	Post	55.8 ± 10.4	56.9 ± 11.8

aBMD: Areal Bone Mineral Density
BMC: Bone Mineral Content
BFLBM: Bone Free Lean Body Mass
* Significantly different than Pre $p \leq 0.05$

Regional aBMD and body composition information is in Table 3. No significant group × time interactions, or main effects for time or group were found for arm BMC, leg BMC, or leg BFLBM (all $p \geq 0.309$). A significant time effect was found as arms % fat and fat mass significantly decreased from pre to mid testing periods (both $p \leq 0.047$). Arm BFLBM significantly increased from pre to mid and from pre to post testing periods (both $p \leq 0.047$). Legs % fat and fat mass also decreased from pre to mid testing periods but returned to baseline values at the post testing period (both $p \leq 0.018$).

Table 3. Regional aBMD and Body Composition Over Time (means ± SD).

	Time	**ROTC (n=18)**	**Controls (n=18)**
Arms BMC (g)	Pre	434.5 ± 106.1	418.4 ± 99.8
	Mid	435.4 ± 102.2	419.7 ± 98.5
	Post	435.6 ± 107.3	417.0 ± 97.0
Arms % Fat	Pre	18.4 ± 7.0	19.5 ± 7.8
	Mid *	17.9 ± 7.1	18.9 ± 7.3
	Post *	17.9 ± 7.2	18.9 ± 7.4
Arms Fat Mass (kg)	Pre	1.7 ± 0.5	1.7 ± 0.4
	Mid *	1.6 ± 0.5	1.7 ± 0.4
	Post *	1.6 ± 0.5	1.6 ± 0.4
Arms BFLBM (kg)	Pre	7.4 ± 2.0	7.4 ± 2.4
	Mid *	7.5 ± 1.9	7.4 ± 2.4
	Post ‡	7.5 ± 2.1	7.4 ± 2.4
Legs BMC (g)	Pre	1139.1 ± 218.9	1090.0 ± 223.9
	Mid	1139.1 ± 215.1	1111.0 ± 219.4
	Post	1140.1 ± 221.0	1133.7 ± 226.4
Legs % Fat	Pre	23.5 ± 6.5	23.6 ± 8.1
	Mid *	23.0 ± 6.8	22.6 ± 8.1
	Post	22.8 ± 6.5	23.3 ± 7.7
Legs Fat Mass (kg)	Pre	6.0 ± 1.7	6.1 ± 1.6
	Mid *	5.9 ± 1.6	5.8 ± 1.5
	Post	5.9 ± 1.7	5.9 ± 1.4
Legs BFLBM (kg)	Pre	18.7 ± 3.5	18.7 ± 4.6
	Mid	18.9 ± 3.6	20.0 ± 5.8
	Post	19.0 ± 3.6	19.4 ± 4.5

BMC: Bone Mineral Content
BFLBM: Bone Free Lean Body Mass
*Significantly different than Pre $p \leq 0.05$
‡Significantly different than Mid $p \leq 0.05$

Lumbar Spine (L1-L4) and dual hip aBMD and BMC variables are displayed in Tables 4 and 5. No significant group × time interactions, or main effects for time or group were found for any non-dominant hip variables or dominant trochanter aBMD, femoral neck BMC, or trochanter BMC (all $p \geq 0.059$). Significant group × time interactions were found for the spine, dominant femoral neck, and dominant total hip aBMD and BMC (all $p \leq 0.033$). However, post hoc analysis showed that the spine aBMD was not significantly different between time points within these groups (both $p \geq 0.076$). Dominant femoral neck aBMD significantly increased in ROTC and decreased in controls (both $p \leq 0.024$). The dominant total hip aBMD significantly increased in ROTC, $p=0.017$; while, dominant total hip BMC significantly decreased in controls, $p=0.020$. Figure 3 shows the significant interaction effect for dominant total hip aBMD.

Table 4. Lumbar Spine and Dual Hip aBMD (g/cm^2) Over Time (means ± SD).

	Time	**ROTC (n=18)**	**Controls (n=18)**
Lumbar Spine L1-L4	Pre	1.305 ± 0.110	1.325 ± 0.162
	Post †	1.312 ± 0.117	1.318 ± 0.153
Dominant			
Femoral Neck	Pre	1.219 ± 0.136	1.244 ± 0.154
	Post †	1.229 ± 0.143*	1.232 ± 0.154*
Trochanter	Pre	0.979 ± 0.122	0.963 ± 0.149
	Post	0.982 ± 0.122	0.954 ± 0.148
Total Hip	Pre	1.202 ± 0.134	1.200 ± 0.163
	Post †	1.210 ± 0.132*	1.197 ± 0.163
Non-Dominant			
Femoral Neck	Pre	1.220 ± 0.137	1.237 ± 0.169
	Post	1.221 ± 0.137	1.239 ± 0.167
Trochanter	Pre	0.976 ± 0.122	0.985 ± 0.178
	Post	0.982 ± 0.123	0.973 ± 0.158
Total Hip	Pre	1.199 ± 0.134	1.199 ± 0.175
	Post	1.203 ± 0.130	1.200 ± 0.169

† Significant Group × Time Interaction $p \leq 0.05$ *Significantly different than Pre $p \leq 0.05$

Table 5. Lumbar Spine and Dual Hip BMC (g) Over Time (means ± SD).

	Time	ROTC (n=18)	Controls (n=18)
Lumbar Spine L1-L4	Pre	75.7 ± 14.0	83.0 ± 18.3
	Post	77.3 ± 12.8	83.2 ± 18.0
Dominant			
Femoral Neck	Pre	6.3 ± 1.0	6.3 ± 1.1
	Post	6.3 ± 1.1	6.2 ± 1.1
Trochanter	Pre	14.1 ± 3.2	12.9 ± 3.8
	Post	14.2 ± 3.2	12.7 ± 3.9
Total Hip	Pre	41.4 ± 6.7	40.2 ± 7.8
	Post †	41.6 ± 6.8	39.8 ± 7.7*
Non-Dominant			
Femoral Neck	Pre	6.3 ± 1.1	6.3 ± 1.1
	Post	6.3 ± 1.0	6.3 ± 1.2
Trochanter	Pre	14.1 ± 2.7	13.3 ± 4.1
	Post	14.3 ± 2.8	13.4 ± 4.1
Total Hip	Pre	41.5 ± 6.4	40.6 ± 8.1
	Post	41.7 ± 6.5	40.6 ± 8.0

† Significant Group × Time Interaction p≤0.05 *Significantly different than Pre p≤0.05

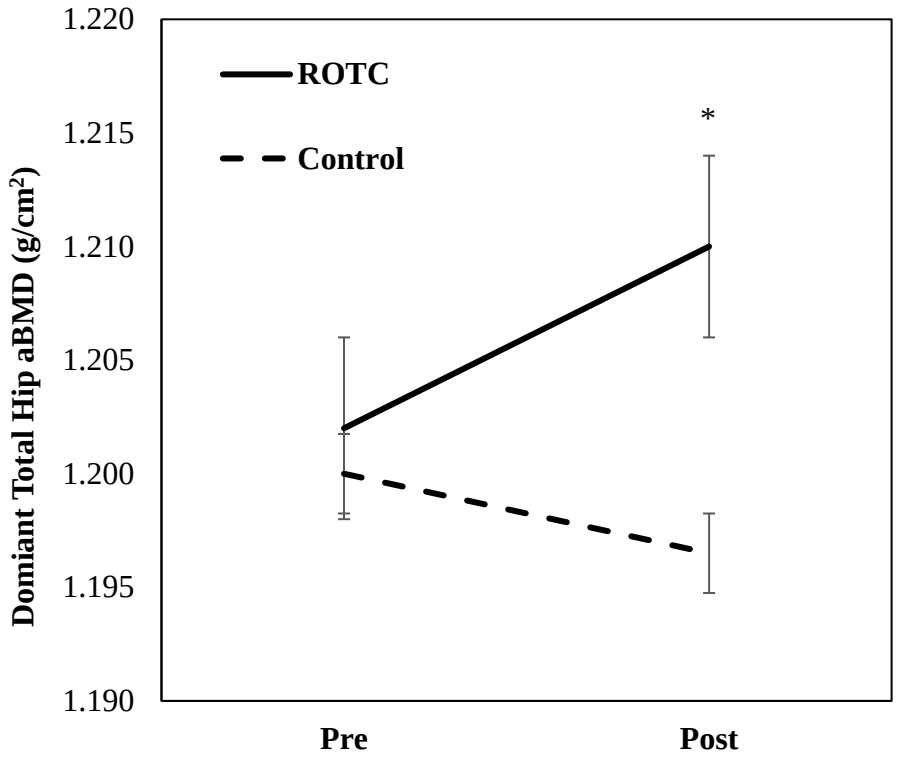


Figure 3. Dominant Total Hip aBMD Group Responses Over Time (means ±SE).
* Significantly different than Pre p≤0.05

No significant group × time interactions, or main effects for time or group were found in either the dominant or non-dominant hip strength index, buckling ratio, section modulus, or cross-section moment of inertia (all $p \geq 0.180$) as shown in Table 6.

Table 6. Hip Structural Analysis Variables Over Time (means ± SD).

	Time	ROTC (n=18)	Controls (n=18)
Dominant Hip			
Strength Index	Pre	1.8 ± 0.3	1.6 ± 0.4
	Post	1.7 ± 0.3	1.7 ± 0.4
Buckling Ratio	Pre	2.8 ± 1.2	2.8 ± 1.5
	Post	2.5 ± 0.8	3.0 ± 1.3
Section Modulus (mm^3)	Pre	925.5 ± 228.2	885.4 ± 188.6
	Post	919.0 ± 248.5	876.0 ± 180.2
CSMI (mm^4)	Pre	15550 ± 5441	14116 ± 3998
	Post	15363 ± 5715	14060 ± 3812
Non-Dominant Hip			
Strength Index	Pre	1.8 ± 0.3	1.7 ± 0.3
	Post	1.7 ± 0.3	1.7 ± 0.3
Buckling Ratio	Pre	2.7 ± 1.4	3.0 ± 1.3
	Post	2.4 ± 1.0	3.0 ± 1.4
Section Modulus (mm^3)	Pre	905.8 ± 216.8	913.9 ± 214.6
	Post	906.1 ± 220.6	908.4 ± 198.8
CSMI (mm^4)	Pre	15373 ± 5582	14953 ± 4602
	Post	15569 ± 5350	14918 ± 4704

CSMI: Cross-Section Moment of Inertia

Peripheral Quantitative Computed Tomography Measures

Tables 7-9 depict the changes over time in pQCT variables from the 4%, 38%, and 66% non-dominant tibia sites. No significant group × time interactions, or main effects for time or group were found for any of the variables at the 4% site (all $p \geq 0.158$), including total and trabecular BMC, vBMD, area, bone strength index, and the periosteal circumference as shown in Table 7.

Table 7. 4% Non-Dominant Tibia pQCT Variables Over Time (means ± SD).

	Time	ROTC (n=18)	Controls (n=18)
Total			
BMC (mg/mm)	Pre	395.0 ± 64.9	398.2 ± 74.9
	Post	394.9 ± 64.8	398.2 ± 74.6
vBMD (mg/cm³)	Pre	344.333 ± 27.703	357.006 ± 37.258
	Post	344.806 ± 28.178	355.722 ± 36.898
Area (mm²)	Pre	1149.44 ± 181.15	1118.84 ± 200.20
	Post	1147.24 ± 179.62	1123.66 ± 203.71
BSI (mg^2/mm^4)	Pre	136.6 ± 28.3	143.2 ± 36.4
	Post	136.8 ± 28.6	142.6 ± 36.1
Trabecular			
BMC (mg/mm)	Pre	324.4 ± 61.6	314.9 ± 68.8
	Post	323.5 ± 60.6	315.9 ± 68.6
vBMD (mg/cm³)	Pre	308.572 ± 27.110	312.572 ± 32.326
	Post	308.594 ± 26.937	311.728 ± 32.714
Area (mm²)	Pre	1051.53 ± 175.80	1008.36 ± 195.50
	Post	1048.34 ± 173.50	1014.97 ± 198.65
BSI (mg^2/mm^4)	Pre	100.9 ± 25.1	99.4 ± 29.7
	Post	100.6 ± 24.8	99.5 ± 29.6
Periosteal Circ. (mm)	Pre	119.8 ± 9.6	118.1 ± 10.5
	Post	119.7 ± 9.5	118.4 ± 10.7

BMC: Bone Mineral Content
vBMD: Volumetric Bone Mineral Density
BSI: Bone Strength Index
Circ: Circumference

No significant group × time interactions, or main effects for time or group were found for any of the variables at the 38% site (all $p \geq 0.110$), including total and cortical BMC, vBMD, area, cortical thickness, periosteal and endosteal circumference, iPolar and stress strain index (SSI) as shown in Table 8.

Table 8. 38% Non-Dominant Tibia pQCT Variables Over Time (means ± SD).

	Time	ROTC (n=18)	Controls (n=18)
Total			
BMC (mg/mm)	Pre	396.7 ± 53.1	411.8 ± 67.3
	Post	398.0 ± 54.0	411.9 ± 67.1
vBMD (mg/cm³)	Pre	934.744 ± 55.116	945.806 ± 65.410
	Post	936.000 ± 54.684	945.972 ± 65.365
Area (mm²)	Pre	425.82 ± 62.19	439.54 ± 88.59
	Post	426.62 ± 62.94	439.66 ± 88.76
Cortical			
BMC (mg/mm)	Pre	381.3 ± 49.9	394.7 ± 62.9
	Post	382.4 ± 50.7	394.7 ± 62.3
vBMD (mg/cm³)	Pre	1175.994 ± 25.824	1171.267 ± 28.178
	Post	1176.206 ± 24.908	1171.083 ± 28.691
Area (mm²)	Pre	324.73 ± 45.16	338.12 ± 60.10
	Post	325.56 ± 45.51	338.20 ± 59.40
Thickness (mm)	Pre	5.99 ± 0.59	6.19 ± 0.69
	Post	6.00 ± 0.59	6.19 ± 0.66
Periosteal Circ. (mm)	Pre	73.0 ± 5.4	73.9 ± 7.6
	Post	73.0 ± 5.5	74.0 ± 7.7
Endosteal Circ. (mm)	Pre	35.3 ± 5.0	35.0 ± 7.0
	Post	35.3 ± 4.9	35.0 ± 7.1
iPolar (mm^4)	Pre	31230.1 ± 8436.4	33066.9 ± 11996.2
	Post	31356.3 ± 8405.0	33105.3 ± 12082.0
SSI (mm³)	Pre	1910.8 ± 385.4	2013.1 ± 532.5
	Post	1911.7 ± 382.5	2024.8 ± 536.8

BMC: Bone Mineral Content
vBMD: Volumetric Bone Mineral Density
Circ: Circumference
SSI: Stress Strain Index

No significant group × time interactions, or main effects for time or group were found for most variables at the 66% site (all $p \geq 0.213$), including total and cortical vBMD and area, cortical BMC and thickness, periosteal and endosteal circumference, iPolar, and SSI as shown in Table 9. Significant time effects were found for 66% total BMC and mCSA which significantly increased from pre to post testing periods (both $p \leq 0.018$).

Table 9. 66% Non-Dominant Tibia pQCT Variables Over Time (means ± SD).

	Time	ROTC (n=18)			Controls (n=18)		
Total							
BMC (mg/mm)	Pre	435.3	±	61.0	450.3	±	74.0
	Post *	436.3	±	61.2	450.9	±	74.1
vBMD (mg/cm³)	Pre	714.772	±	87.558	725.839	±	68.123
	Post	698.367	±	50.828	726.878	±	67.921
Area (mm²)	Pre	619.49	±	118.20	625.93	±	117.94
	Post	627.71	±	97.55	625.82	±	117.73
Cortical							
BMC (mg/mm)	Pre	396.5	±	53.9	407.4	±	64.6
	Post	397.1	±	56.4	408.2	±	65.1
vBMD (mg/cm³)	Pre	1136.906	±	25.431	1136.122	±	25.080
	Post	1136.800	±	21.860	1136.839	±	26.351
Area (mm²)	Pre	349.33	±	50.44	359.57	±	62.36
	Post	349.86	±	52.38	360.03	±	62.62
Thickness (mm)	Pre	4.83	±	0.52	4.93	±	0.64
	Post	4.74	±	0.53	4.94	±	0.63
Periosteal Circ. (mm)	Pre	87.8	±	8.9	88.3	±	8.6
	Post	88.6	±	7.0	88.3	±	8.5
Endosteal Circ. (mm)	Pre	57.5	±	9.9	57.3	±	8.3
	Post	58.7	±	6.5	57.2	±	8.2
iPolar (mm^4)	Pre	57755.4	±	17937.8	58221.3	±	19431.4
	Post	58624.3	±	16222.0	58224.2	±	19350.8
SSI (mm³)	Pre	2904.9	±	701.7	2994.2	±	745.3
	Post	2939.8	±	621.8	2991.7	±	750.3
Muscle CSA (mm²)	Pre	7534.3	±	1038.9	7972.4	±	1736.1
	Post *	7734.1	±	1049.7	8043.4	±	1789.3

BMC: Bone Mineral Content
vBMD: Volumetric Bone Mineral Density
Circ: Circumference
SSI: Stress Strain Index
* Significantly different than Pre $p \leq 0.05$

Biomarkers

Blood samples were allowed to clot, then centrifuged to separate the serum from the red blood cells, and frozen until assays were performed. Microtubes and all kit components were allowed to reach room temperature and were then assayed in duplicate. Instructions for the assay kits for PTH and sclerostin were followed exactly. PTH had two statistical outliers (greater than two box plots) (ROTC n=1; control n=1) and sclerostin had one statistical outlier (ROTC n=1). Exclusion of outliers did not change any statistical outcomes so subsequent analysis included outliers. Table 10 shows the biomarker responses over time for each group. No sex differences between either biomarker responses or %Δ were found when the whole group was considered, or when ROTC and controls were considered separately (all $p \geq 0.138$).

Table 10. Biomarker Responses Over Time (means ± SD).

	Time	**ROTC (n=18)**	**Controls (n=18)**
Parathyroid Hormone (pg/mL)	Pre	40.85 ± 22.52	47.77 ± 19.52
	Post	46.61 ± 22.37	45.78 ± 16.01
Sclerostin (ng/mL)	Pre	0.420 ± 0.137	0.393 ± 0.097
	Post	0.405 ± 0.122	0.386 ± 0.115

Parathyroid Hormone

No significant group × time interactions, or main effects for time or group were found (all $p \geq 0.323$) as shown in Table 10 and Figure 4. Mean PTH percent changes were 42.4% and -3.9% for ROTC and controls respectively ($p=0.152$) as displayed in Figure 5.

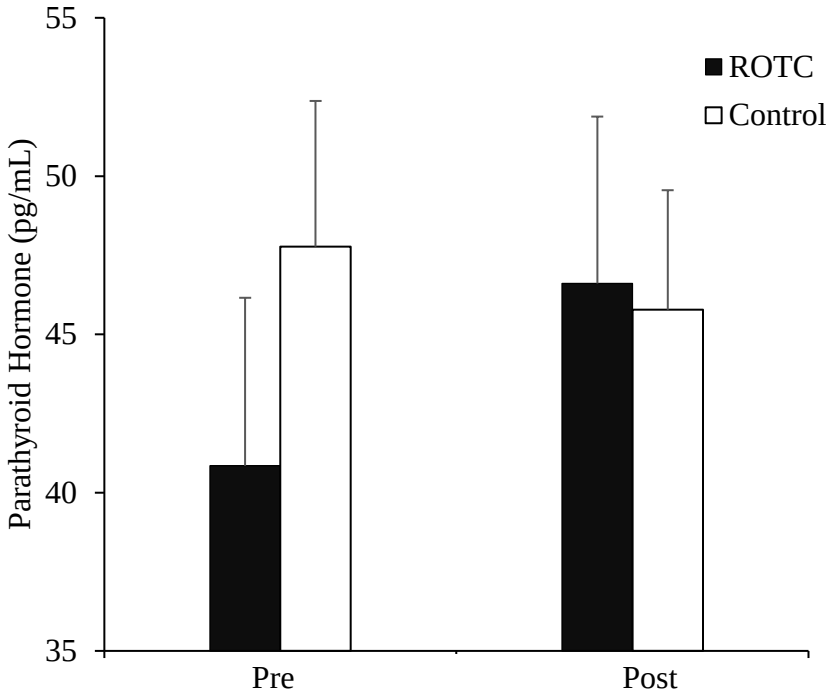


Figure 4. Serum Parathyroid Hormone Concentrations (means ± SE).

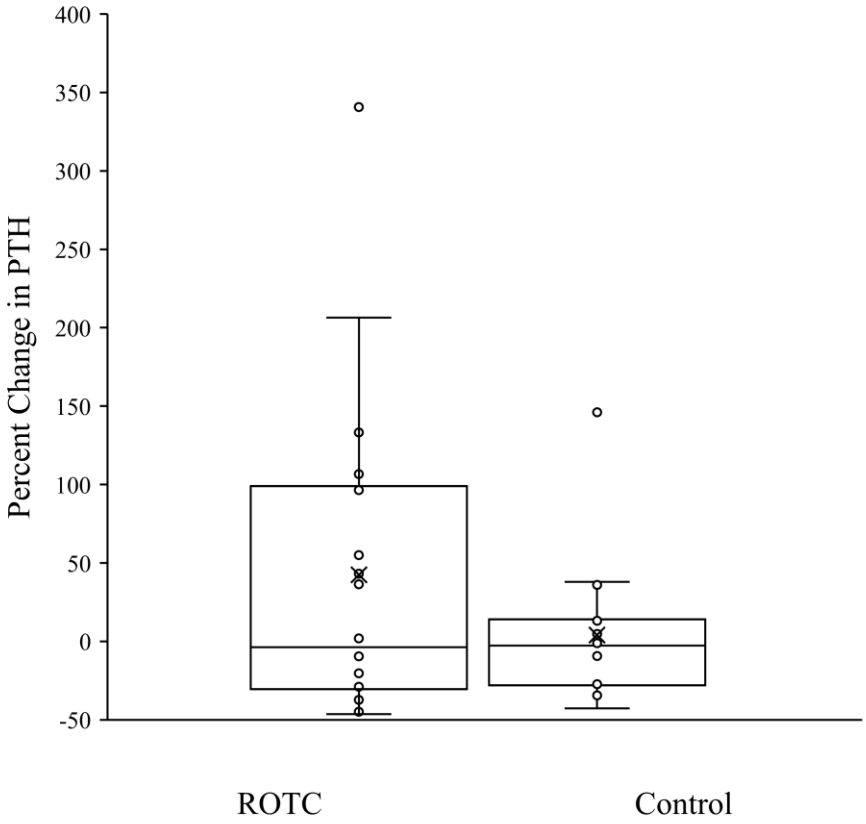


Figure 5. Percent Changes in Parathyroid Hormone Concentrations (means ± 95% CI). X denotes mean; line denotes median; boxes denote the 75th and 25th quartiles.

Sclerostin

Figure 6 displays the changes over time in the serum sclerostin concentrations for each group. No significant group × time interactions, or main effects for time or group were found (all $p \geq 0.189$) as shown in Table 10. Mean sclerostin percent changes were -2.7 % and -2.1% in ROTC and controls respectively ($p=0.501$) as shown in Figure 7.

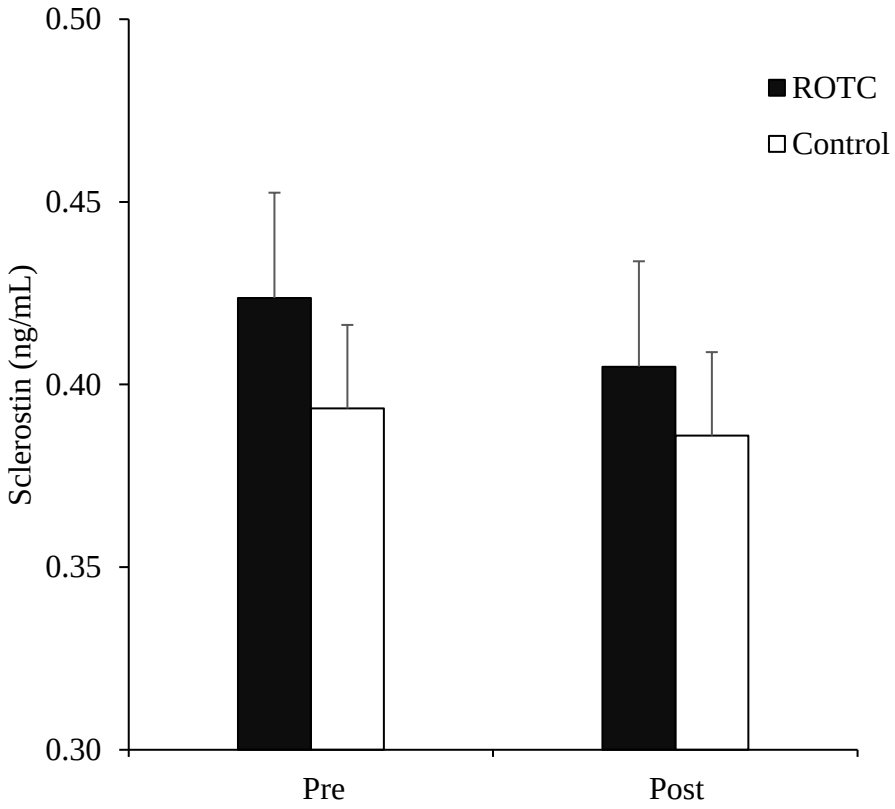


Figure 6. Serum Sclerostin Concentrations (means ± SE).

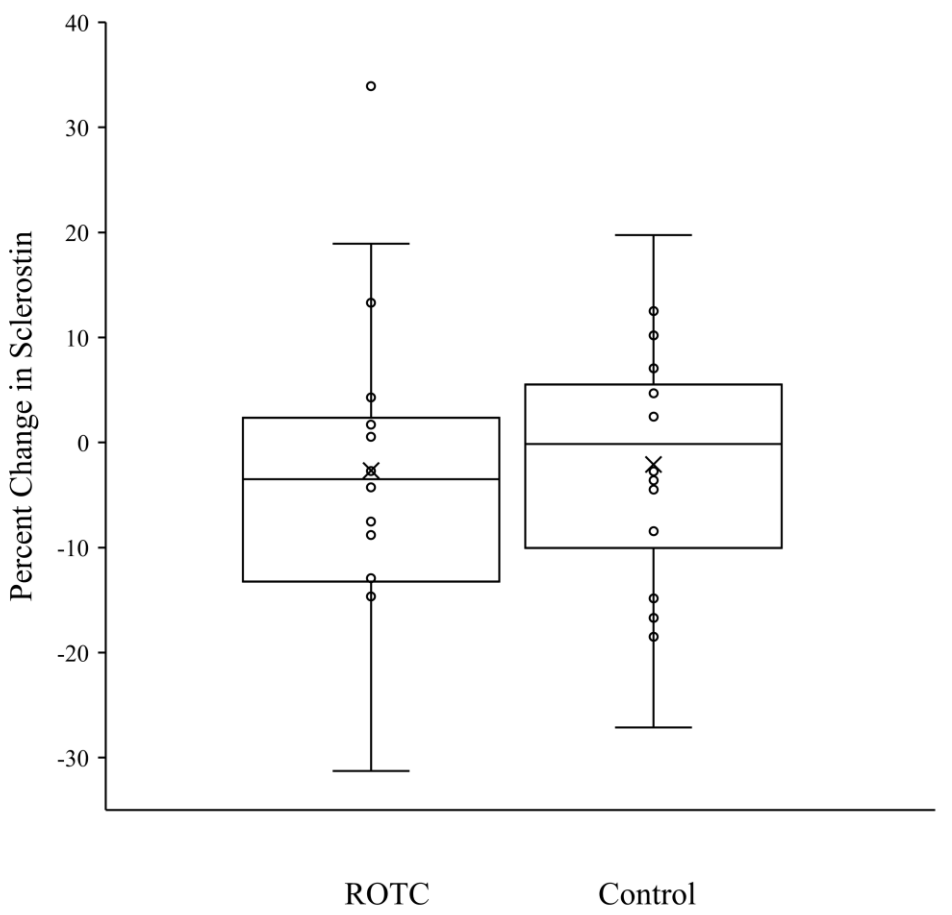


Figure 7. Percent Changes in Sclerostin Concentrations (means ± 95% CI).
X denotes mean; line denotes median; boxes denote the 75th and 25th quartiles.

Physical Performance Measures

No significant group × time interactions were found for any of the muscle strength and power measures (all $p \geq 0.143$); however, significant time effects were found as shown in Table 11. No significant changes occurred in the right hand grip strength; however, left hand grip strength decreased significantly at the post testing period. Both jump height and time in the air and bench press and leg press increased from pre to mid and leveled off at the post testing period (all $p \leq 0.024$). Jump power and velocity increased pre to mid, however, returned to baseline by the post testing period (all $p \leq 0.003$).

Table 11. Muscular Strength and Power Measures Over Time (means ± SD).

	Time	ROTC (n=18)	Controls (n=18)
Right Hand Grip (kg)	Pre	48.2 ± 10.6	46.4 ± 10.1
	Mid	48.2 ± 10.5	47.2 ± 11.6
	Post	46.6 ± 11.0	45.9 ± 11.0
Left Hand Grip (kg)	Pre	46.3 ± 11.6	46.1 ± 12.1
	Mid	46.1 ± 10.8	47.5 ± 13.8
	Post * ‡	43.8 ± 9.5	45.2 ± 13.5
Jump Height (cm)	Pre	48.5 ± 9.9	51.1 ± 11.5
	Mid *	50.5 ± 9.1	53.3 ± 12.7
	Post *	49.5 ± 9.4	52.3 ± 12.7
Time in Air (sec)	Pre	0.62 ± 0.07	0.62 ± 0.07
	Mid *	0.62 ± 0.07	0.65 ± 0.07
	Post *	0.62 ± 0.07	0.62 ± 0.07
Jump Power (w)	Pre	978.3 ± 215.7	1024.7 ± 261.9
	Mid *	997.4 ± 222.1	1077.4 ± 259.7
	Post ‡	979.3 ± 235.2	1025.7 ± 279.6
Jump Velocity (m/s)	Pre	1.4 ± 0.2	1.4 ± 0.2
	Mid *	1.4 ± 0.2	1.4 ± 0.2
	Post ‡	1.3 ± 0.2	1.4 ± 0.2
Leg Press (kg)	Pre	251.9 ± 80.4	257.1 ± 106.8
	Mid *	277.6 ± 81.8	283.6 ± 109.2
	Post *	283.7 ± 80.7	284.9 ± 112.2
Bench Press (kg)	Pre	80.0 ± 30.6	77.9 ± 36.0
	Mid *	83.7 ± 32.2	81.7 ± 38.3
	Post *	82.8 ± 30.0	80.6 ± 35.0

* Significantly different than Pre $p \leq 0.05$

‡ Significantly different than Mid $p \leq 0.05$

No significant group × time interactions were found for any of the aerobic capacity testing variables as shown in Table 12 (all $p \geq 0.087$). ROTC had greater pre and post relative VO_2 peak as compared to the control group ($p=0.014$), while both groups significantly increased over time ($p=0.007$). Additional significant main effects for time were found as absolute VO_2 and total time to exhaustion significantly increased while respiratory exchange ratio (RER) significantly decreased (all $p \leq 0.022$).

Table 12. Aerobic Capacity Testing Measures Over Time (means ± SD).

	Time	ROTC (n=18)	Controls (n=18)
Absolute VO_2 Peak (L/min)	Pre	3.86 ± 0.97	3.59 ± 0.77
	Post *	3.98 ± 0.98	3.68 ± 0.80
Relative VO_2 Peak (kg/mL/min)	Pre	52.5 ± 7.8 #	47.1 ± 4.8
	Post *	53.8 ± 8.1 #	48.5 ± 5.6
Respiratory Exchange Ratio	Pre	1.15 ± 0.06	1.16 ± 0.07
	Post *	1.14 ± 0.07	1.12 ± 0.04
Maximum Heart Rate (bpm)	Pre	195 ± 8	196 ± 7
	Post	195 ± 7	198 ± 8
RPE	Pre	18.1 ± 1.3	18.4 ± 1.4
	Post	18.2 ± 1.1	18.8 ± 0.9
Time to Exhaustion (min)	Pre	10.5 ± 1.3	10.1 ± 1.7
	Post *	11.6 ± 1.4	10.9 ± 1.7

RPE: Respiratory Exchange Ratio

* Significantly different than Pre $p \leq 0.05$

Significantly different than Controls $p \leq 0.05$

Correlations between Biomarkers and Dependent Variables

The secondary purpose of this study was to determine the relationship between PTH and sclerostin with bone variables. Few significant associations between PTH and DXA or pQCT were found for the whole group or when ROTC and control groups were considered separately. Pre and post non-dominant hip CSMI was positively correlated with PTH (both $p \leq 0.029$; Pearson's r ranged from 0.365-0.371) when the whole group was considered. In ROTC participants pre and post dominant hip bucking ratio was negatively correlated with PTH (both $p \leq 0.024$; Pearson's r ranged from 0.529 - 0.541). Correlations between the pre and post PTH and pre and post total BSI, 4% trabecular BSI, 38% SSI, and 66% SSI were all non-significant (all $p \geq 0.323$) as shown in Table 13.

Table 13. Correlation Matrix (r) for PTH and Bone Strength/Strain Indices Pre and Post Intervention.

	Time	4% Total BSI	4% Trabecular BSI	38% SSI	66% SSI
PTH (pg/mL)	Pre	-.129	-.010	-.055	-0.088
PTH (pg/mL)	Post	-.008	.081	.017	0.125

BSI: Bone Strength Index SSI: Stress-Strain Index

Sclerostin was significantly correlated with many DXA and pQCT variables. When correlating DXA variables and sclerostin for the ROTC participants, most of the 86 variables considered were significantly associated. In controls, only five of the 86 DXA variables were correlated ($p \leq 0.047$; Pearson's r ranged from 0.475 – 0.529). In ROTC almost 75% of all pQCT variables were positively correlated with sclerostin (all $p \leq 0.039$; Pearson's r ranged from 0.494 - 0.775), while in controls only nine of the 64 pQCT variables were correlated ($p \leq 0.047$; Pearson's r ranged from 0.474 – 0.547). Additionally, Table 14 displays the relationship between both pre and post serum sclerostin concentrations and all pre and post pQCT measures of bone strength (all $p \leq 0.036$).

Table 14. Correlation Matrix (r) for Sclerostin and Bone Strength/Strain Indices Pre and Post Intervention.

	Time	4% Total BSI	4% Trabecular BSI	38% SSI	66% SSI
Sclerostin (ng/mL)	Pre	.444**	.351*	.401*	.533**
Sclerostin (ng/mL)	Post	.434**	.355*	.407*	.514**

BSI: Bone Strength Index * Significant correlations, $p < 0.05$
SSI: Stress-Strain Index ** Significant correlations, $p < 0.01$

The last aim of the study included investigating the relationships between body composition, muscular strength and power measures, and aerobic fitness and the biomarkers, PTH and sclerostin. PTH was not associated with any fat mass, % fat,

BFLBM or 66% tibia mCSA measures for either group (all $p \geq 0.104$; Pearson's r ranged from -0.395 to 0.147). Pre PTH was a positive correlate of pre relative VO_2 peak ($p=0.031$; Pearson's $r=0.510$) but was negatively correlated with pre heart rate and rating of perceived exertion (RPE) (both $p \leq 0.014$; Pearson's r was between -0.581 and -0.587).

Sclerostin was significantly correlated with many body composition and performance measures, which were group dependent. Total body BFLBM was significantly positively correlated with sclerostin (Figure 8); while no significant correlations between fat mass (Figure 9) for sclerostin were found for either group. Pre and post sclerostin concentrations were not significantly correlated with pre or post total %BF as shown in Figures 12 and 13 in Appendix H (both $p=0.051$). When correlating performance variables and sclerostin, ROTC had nearly two times more significant positive correlates as compared to controls (all $p \leq 0.042$; Pearson's r ranged from 0.498 - 0.804). Bench press and sclerostin demonstrated the strongest positive association ($p \leq 0.001$; Pearson's $r = 0.804$) as shown in Figure 16 in Appendix H, while jump power was the only jump variable correlated with sclerostin for any group, at any time point (all $p \leq 0.015$; Pearson's r ranged from 0.565 to 0.676) as shown in Figure 14 in Appendix H.

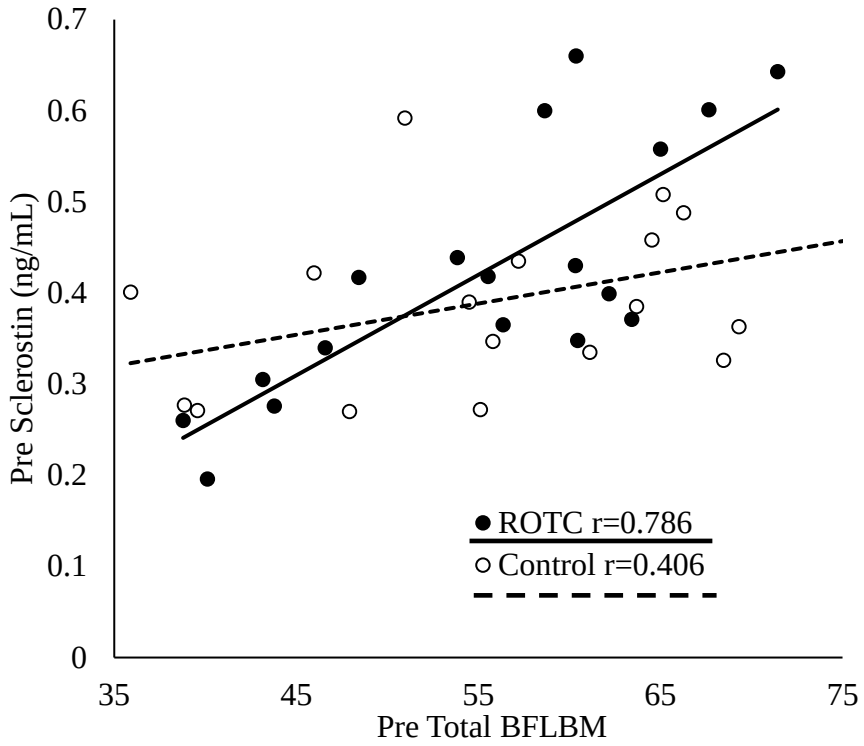


Figure 8. Correlation Between Baseline Sclerostin and Total BFLBM.

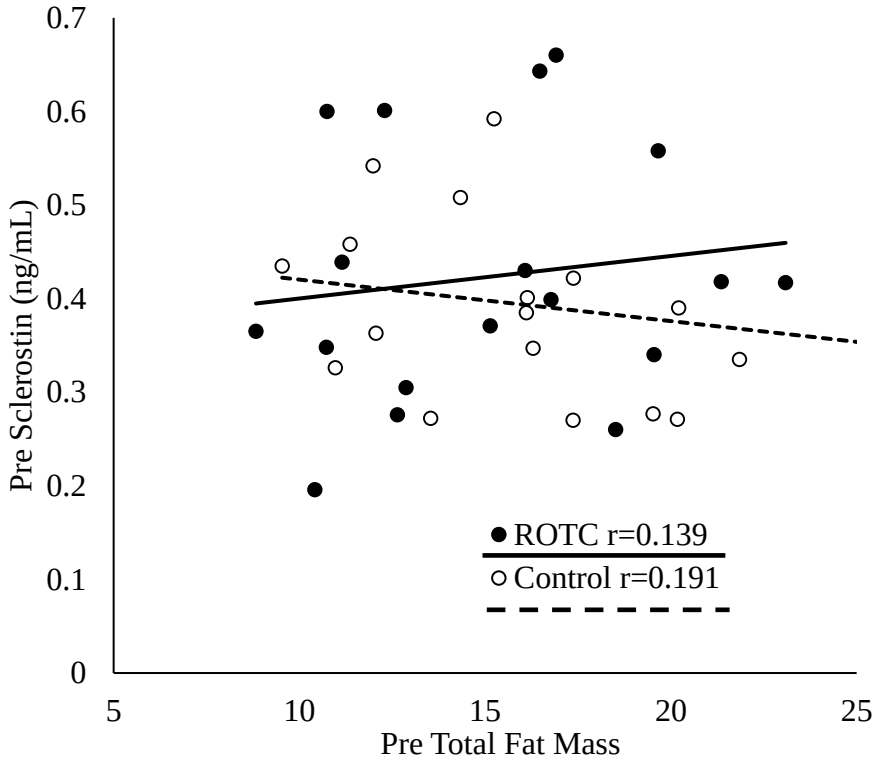


Figure 9. Correlation Between Baseline Sclerostin and Total Fat Mass.

As previously noted in Tables 13 and 14, PTH had very little association with measures of tibial bone strength while sclerostin was strongly correlated. Similar strong correlations were found between calf muscle cross-sectional area (mCSA) at the 66% site and measures of bone strength. Pre and post mCSA was significantly positively correlated with bone strength variables at all three tibia sites (all ≤0.003), as shown in Table 15.

Table 15. Correlation Matrix (r) for mCSA and Bone Strength/Strain Indices Pre and Post Intervention.

	Time	4% Total BSI	4% Trabecular BSI	38% SSI	66% SSI
mCSA (mm^2)	Pre	0.581**	0.482**	0.641**	0.604**
mCSA (mm^2)	Post	0.607**	0.522**	0.653**	0.640**

mCSA: Muscle Cross-Sectional Area
BSI: Bone Strength Index
SSI: Stress-Strain Index
** Significant correlations, p≤ 0.01

Regression Analyses for Biomarkers and Measures of Bone Density and Quality

PTH and sclerostin were chosen as biomarkers due to the poor injury prediction capabilities of BTM in military personnel reported in the literature. Simple linear regression was used to identify if each marker demonstrated predictive capabilities for the dependent variables total body aBMD, lumbar spine aBMD, hip aBMD and measures of tibia bone strength. Table 16 shows the model outputs for each biomarker separately with the previously mentioned dependent variables. Both dependent and independent variables used in the regression models were calculated percent changes (%Δ) from pre to post intervention period.

Table 16. Regression Models for %Δ in Biomarkers and Bone Density and Quality.

Dependent Variable	Independent Variables	β	SEE	R^2
Total Body aBMD	Parathyroid Hormone	-0.426	2.63681	0.182*
	Sclerostin	0.572	0.096995	0.327**
Lumbar Spine L1-L4 aBMD	Parathyroid Hormone	-0.083	1.5666	0.007
	Sclerostin	0.012	1.5719	0.000
Dominant Femoral Neck aBMD	Parathyroid Hormone	-0.067	1.8065	0.005
	Sclerostin	-0.12	1.7884	0.014
Dominant Hip aBMD	Parathyroid Hormone	0.193	1.0772	0.037
	Sclerostin	-0.116	1.1404	0.014
4% Total BSI	Parathyroid Hormone	-0.077	1.91646	0.006
	Sclerostin	-0.047	1.90562	0.002
4% Trabecular BSI	Parathyroid Hormone	0.167	2.20009	0.028
	Sclerostin	0.053	2.22454	0.003
38% SSI	Parathyroid Hormone	-0.175	1.26096	0.031
	Sclerostin	-0.333	1.2373	0.111*
66% SSI	Parathyroid Hormone	-0.102	6.23964	0.011
	Sclerostin	0.099	0.099	0.010

* Significant $p \leq 0.05$
** Significant $p \leq 0.01$

Sclerostin significantly predicted 38% SSI as shown above. In Table 17, other potential predictors were added to investigate their potential predictive power. These independent variables are common field measures of performance or body composition. %Δ total BF%, VO_2 peak, leg press, bench press, and jump power were not independently significantly correlated with the %Δ in 38% SSI. Multiple linear regression using the stepwise method was used to combine independent variables into predictive models. When the performance measures VO_2 peak, leg press, bench press, and jump power were combined into a single predictive model they did not predict a significant about of the variance of %Δ in 38% SSI as shown in Table 18 (p=0.178). When sclerostin and 66% mCSA were combined into a predictive model they predicted nearly 45% of the variance of %Δ in 38% SSI as shown in Table 19 (p=0.003).

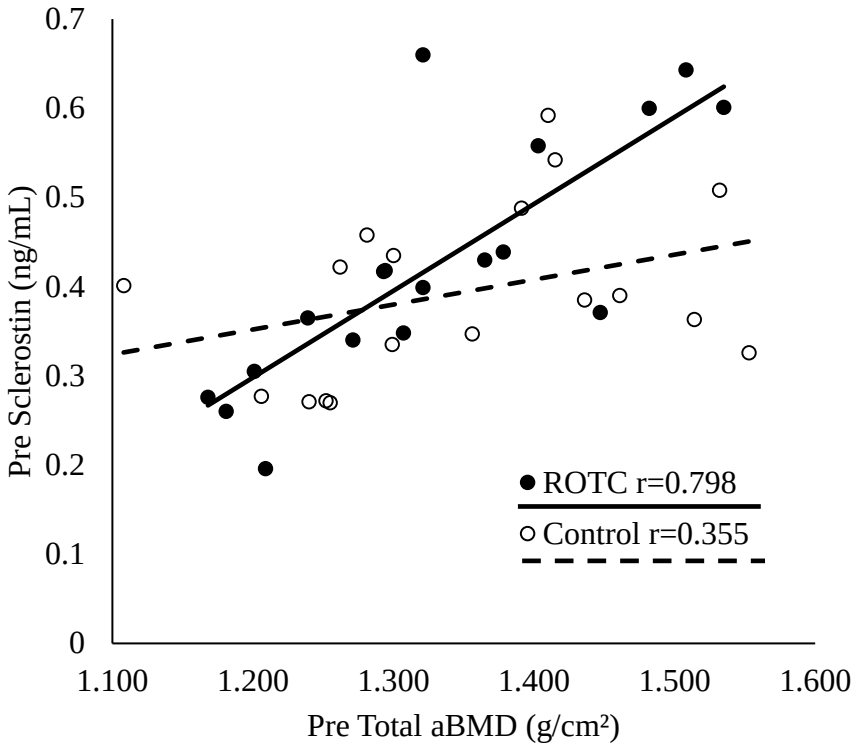


Figure 10. Correlation between Baseline Sclerostin and Total aBMD.

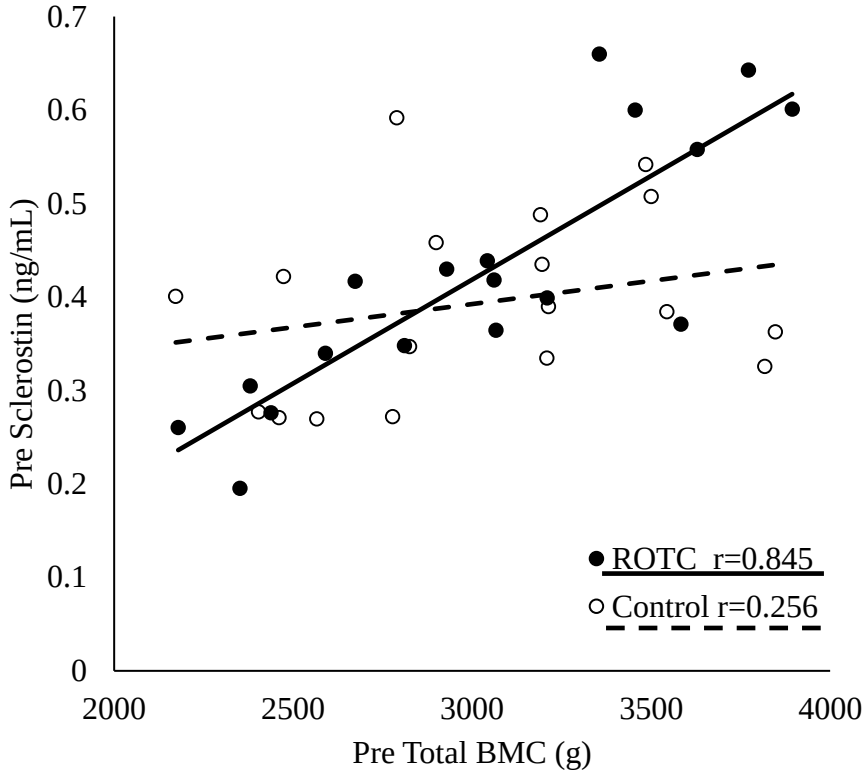


Figure 11. Correlation between Baseline Sclerostin and Total BMC.

Table 17. Potential Predictors of %Δ in 38% SSI.

Dependent Variable	Independent Variables	β	SEE	R^2
38% SSI	Sclerostin	-0.333	1.2373	0.111*
	Body Fat %	-0.177	1.27561	0.031
	VO_2 Peak	0.241	1.25792	0.058
	Leg Press	0.22	1.2989	0.048
	Bench Press	-0.039	1.29497	0.002
	Jump Power	-0.21	1.26714	0.044

* Significant $p \leq 0.05$
** Significant $p \leq 0.01$

Table 18. Predictive Model for %Δ in 38% SSI using Physical Performance Measures.

Dependent Variable	Independent Variables	β	SEE	R^2
38% SSI	VO_2 Peak	0.185	1.2591	0.19
	Leg Press	-0.362		
	Bench Press	0.314		
	Jump Power	-0.038		

Table 19. Predictive Model for %Δ 38% SSI using %Δ Sclerostin and 66% mCSA.

Dependent Variable	Independent Variables	β	SEE	R^2
38% SSI	Sclerostin	0.570	354.36	0.443**
	66% mCSA	0.194		

** Significant $p \leq 0.01$

Discussion

Bone injuries cost the U.S. military over $100 million dollars per year (14) and are considered the leading cause of injury related discharge reported in both USMC and Naval basic training programs (53). Currently, the biomarkers used to predict bone injuries in military cohorts demonstrate poor predictive power and very inconsistent findings (3, 31, 35, 205). The purpose of this study was to investigate Parathyroid Hormone and sclerostin as potential biomarkers of skeletal change during a military training intervention period using college-aged students enrolled in USMC and Naval

ROTC programs. Neither biomarker demonstrated significant changes over time; however, %Δ in both PTH and sclerostin were significantly associated with %Δ in total body aBMD. Sclerostin responses were also significantly correlated with %Δ in 38% SSI. Sclerostin showed many significant positive associations with measures of bone density, quality, and strength, BFLBM, and performance measures. Both groups exhibited significant losses in total BF %. Dominant femoral neck aBMD significantly increased in ROTC and decreased in controls. The dominant total hip aBMD significantly increased in ROTC; while, dominant total hip BMC significantly increased in controls after the eight week intervention.

Parathyroid Hormone Responses

Parathyroid Hormone (PTH), in conjunction with Vitamin D, is a primary regulator of serum calcium ion concentrations and can negatively impact skeletal health if over or under produced (32). Because of this relationship, serum PTH concentrations can be drastically altered by dietary calcium or seasonal effects of Vitamin D (111); unfortunately, many studies investigating military cohorts do not collect any dietary information or account for seasonal variations. In this study, dietary calcium intake was above the recommended 1000 mg/day (203), so calcium deficiency is not expected to affect PTH responses. The control group provided blood draws within two weeks of their matched ROTC participant, which allowed for the characterization of potential seasonal effects.

The lack of group × time interactions in PTH responses described in the current study may have been due to either the short intervention length or the frequency of blood sampling. Lester et al. (6) followed 69 college-aged physically inactive females

across an eight week exercise intervention period. All participants were enrolled in either an aerobic training, resistance training, aerobic plus resistance training, or control group. All groups demonstrated a significant initial rise in PTH levels from pre testing to four weeks; however, levels returned to baseline at the eight week post testing period. The authors did not provide any hypothesis for why this initial rise in PTH was observed for all groups. The current study also demonstrated no change from pre to eight week post testing period; however, since a mid or four week blood draw was not conducted inferences about the early PTH responses can not be made. Evans et al. (35) reported opposite PTH responses in male Israeli Defense Forces recruits as PTH levels significantly decreased after eight weeks of basic training, but returned to baseline levels by the post (16 weeks) testing period. Despite no sex differences in baseline calcium, Vitamin D, or PTH, male recruits exhibited a significant decrease in Vitamin D from pre to midway testing which was correlated with the significant drop in PTH reported by Evans et al. (35); however, they do not speculate as to why this counterintuitive finding emerged.

Valimaki et al. (205) reported that elite Israeli infantry recruits who reported a stress fracture during a 14 week basic training program had mean PTH levels greater than pre or post mean PTH concentrations in the current study (56 pg/mL verses 48 pg/mL). Chronically elevated PTH concentrations can result in excess calcium being mobilized from the mineral matrix and a subsequent reduction in the structural integrity of the bone, as observed in hyperparathyroidism (206). However, intermittent PTH administration has been shown to stimulate bone formation by prolonging osteoblast survival (207). Falk et al. (38) described PTH responses post exercise in boys and men.

At baseline both boys and men had similar resting values of PTH. Five minutes after a volume and intensity matched plyometrics circuit, both boys and men exhibited a significant positive PTH response, one hour later PTH responses had decreased and by 24 hours post exercise bout, PTH levels had returned to baseline. This study did not mention any correction for plasma volume shifts and the acute PTH responses may be overestimated. Scott et al. (174) demonstrated that PTH increased with a single bout of treadmill running in healthy males; however, these authors note that the PTH response post exercise bout was much smaller than exogenous PTH injections provided in clinical settings to promote bone growth, and should not be considered as similar osteogenic responses. This study also did not mention any adjustment for potential plasma volume shifts.

High serum PTH levels have been found to be associated with stress fracture in military recruits (17, 34); however, contrary findings have been observed in athletic and military populations (31, 35, 177-179). In the current study, PTH was significantly correlated with total body aBMD but not with any skeletal sites that are commonly susceptible to fracture such as the lumbar spine, hip, or tibia. From the lack of consistency in the literature in both athletic and military populations and a small number of significant correlations to any DXA, pQCT, or performance measures in this study, PTH does not appear to provide any additional information to changes in bone over time in this particular cohort.

Sclerostin Responses

If an exercise bout results in mechanical loading that exceeds the minimum strain threshold as described by Frost's mechanostat theory (71), acute bone resorption

will occur followed by prolonged bone formation (1). Osteocytes serve as the primary mechanosensing cells of the skeleton and are responsible for the skeletal response to mechanical stimuli. These cells are also responsible for the secretion of sclerostin. Sclerostin can inhibit bone formation due to the disruption of Wnt signaling pathways resulting in fewer active osteoblasts (208). Animal models consistently demonstrate sclerostin production is reduced with increases in mechanical loading, resulting in bone formation (182, 209); however, the sclerostin response to exercise in humans is less clear.

No longitudinal studies to date have used a military population for the investigation of sclerostin responses to exercise, so inferences must come from other cohorts. Many cross-sectional studies have shown that sclerostin increases post exercise bout (181, 185); however, perhaps this demonstrates the relationship between sclerostin and the brief resorptive state of the bone immediately post-exercise (38). Pickering et al. (183) investigated sclerostin responses to 45 minutes of low-speed treadmill running in sedentary young women. Serum sclerostin increased nearly 45% post exercise bout. Falk et al. (38) characterized pre and post sclerostin responses to a plyometrics circuit in boys and men. At baseline boys had significantly greater resting levels of sclerostin as compared to men; however, only men exhibited a significant increase in sclerostin five minutes post exercise and returned to baseline levels one hour post exercise. It should be noted that neither Pickering et al. (183) or Falk et al. (38) mention any adjustments for plasma volume shifts. A semi-longitudinal study conducted by Grasso et al. (186) followed professional cyclists throughout the Giro d'Italia, which is one of the most prominent road cycling events in the world. Across

the 23 day stage race, serum sclerostin concentrations, which were adjusted for plasma volume shifts, significantly increased at each testing period. However, no samples were collected in the days following the race, so true post exercise-bout inferences can not be made. Nine participants ran an ultra-distance race that averaged 34 hours of race time. Participants exhibited no changes in serum sclerostin from pre to post race; however, three day post race sclerostin levels were significantly reduced (186).

To address the cross-sectional nature of most studies investigating sclerostin responses to exercise, Hinton et al. (42) followed men across two separate progressive 12 month exercise programs. Participants were enrolled in either a resistance training (RT) program or a plyometrics program. The RT program consisted of two sessions per week including exercises that targeted the hips and spine such as squats, bent-over-row, dead lift, military press, lunges, and calf raises. Progression was based off of a 6-week training block, followed by a rest week. At the end of each block one repetition maximal efforts were recorded for all lifts and used to program the next block. For the plyometrics protocol, participants met three times per week and engaged in high-impact and odd loading activities such as single leg jumps in multiple directions, box jumps, hurdles, and squat jumps. Like the RT group, 6-week training blocks were used and at the end of each block maximal vertical jump was measured to track progress. Both groups reported significant increases in total body and lumbar spine aBMD, while serum sclerostin was significantly reduced. The consistent progression of exercise-induced mechanical loading may have been why Hinton and colleagues (42) report a significant reduction in sclerostin as prolonged bone formation occurred. In the current study, ROTC training protocols did incorporate exercise progression; however, due to

the short duration of the intervention most skeletal changes observed were very small and within the error of the DXA or pQCT.

Despite no significant sclerostin response, valuable information can still be gathered from the biomarker's positive associations with bone variables, specifically in ROTC participants. Sclerostin was significantly positively correlated with most of the 86 DXA variables considered. Since sclerostin is produced by bone cells, greater concentrations should be observed in individuals with greater skeletal mass, which may partially be driving the number of significant correlations observed with DXA bone variables. Interestingly, sclerostin was a stronger significant positive predictor of total aBMD and BMC in ROTC as compared to controls (r=0.798 vs. r=0.355 and r=0.845 vs. r=0.256 respectively) despite no significant group differences between total body aBMD or BMC as shown in Figures 10 and 11. Sclerostin was not positively associated with fat mass or %BF; which is contrast to other studies reporting positive correlations between sclerostin and aBMD and FM (232, 233).

Krause et al. (234), reported sclerostin and lean mass are directly related as rodents without sclerostin production capabilities demonstrated reduced lean mass. In the current study, sclerostin was positively associated with BFLBM for both groups, corroborating the findings by Krause et al. (234). The association between sclerostin and BFLBM was group dependent, as the relationship was stronger in ROTC as compared to controls (r=0.786 vs. r=0.406). These associations were also evident when considering measures of muscular power. Baseline measures of sclerostin and bench press and leg press were strongly correlated as shown in Figure 15 and 16 in Appendix H, however, the relationship was stronger in ROTC as compared to controls. Baseline

measures of sclerostin and jump power were significantly correlated in ROTC, but not controls.

Bone density and bone geometry together play a critical role in skeletal integrity (58). Sclerostin was strongly associated with measures of bone geometry and a significant positive predictor of bone strength indices at all tibia sites (R^2 ranged from 0.123-0.284). This is very important to consider because distal tibial fractures are among the most common site in military cohorts (210, 211). The sclerostin response was the only significant predictor of %Δ in 38% SSI, as compared to other commonly measured metrics in military studies, such as total BF% (skinfold calipers), VO_2 peak (estimated from 2 mile run times), bench press, leg press, and jump power. (210, 211). Nearly 45% of %Δ in 38% SSI was predicted by %Δ sclerostin and 66% mCSA. Due to the many positive correlations between sclerostin and bone variables, and the significant predictive capabilities of the biomarker for measures of bone strength, sclerostin should be considered in future studies investigating skeletal changes in this cohort.

Areal Bone Mineral Density and Body Composition

Significant group × time interactions were found for the dominant femoral neck, and dominant total hip aBMD and BMC. Dominant femoral neck aBMD significantly increased in ROTC and decreased in controls. The dominant total hip aBMD significantly increased in ROTC; while, dominant total hip BMC significantly decreased in controls. Although the increases in dominant femoral neck and total hip aBMD in ROTC participants was statistically significant, the magnitude of these changes were less than the CV% of the DXA scanner, and are not considered significant. Controls exhibited a significant decrease in dominant femoral neck aBMD

and in total hip BMC, both %Δ were only slightly above the CV% of the DXA scanner and are not considered clinically significant. Hip Structural Analysis (HSA) uses information gathered from the regional dual femur scans to estimate structural geometry from the planar image. The analysis uses pixel density information and the measured BMC to make inferences about skeletal geometry (212). The significant changes in hip BMC were most likely too small to translate to significant differences in HSA measures. Additionally, significant positive body composition changes were reported in both ROTC and control participants. A significant time effect was observed as total BF% decreased while total body BFLBM increased from pre to mid points in both groups. The magnitude of changes for total BF% exceeded the LSC for the DXA machine, however, the gain in BFLBM did not.

The average %Δ in total BF% was 2.8% for ROTC and controls from pre to mid testing. This study utilized the DXA machine for body composition testing which provides excellent precision values. Many other studies conducted with military cohorts use skinfold measurements to make assumptions about body composition and should be considered with caution. The magnitude of the changes in the current study were nearly three times greater than what was described by Evans et al. (35) who followed 194 Israeli Defense Forces recruits over four months. Evans et al. used skinfold measurements which may have not been a sensitive enough measure to detect small but significant total BF% changes. Using DXA, Armstrong et al. (8) followed 31 incoming freshman (plebes) at the United States Naval Academy in 2000. Baseline characteristics were very similar to the current study's cohort for age, height, body mass, and total body BMC (g); however, the average total BF% was nearly 5% lower

than our cohort (8). These differences are most likely attributable to the frequency and intensity differences between the exercise interventions. Plebes summer basic training was described as 5 days/wk with 8.5 hours/day of physical training or drill training while the current study intervention was only 2 days/week with 2-3 hours/day of physical training or drill training.

No significant changes were observed in total aBMD for either group. This may have been due to the short duration of the intervention. Ahola et al. (213) followed women for 12 months with serial DXA scans to investigate the time course of exercise induced aBMD changes. Only women who reported high levels of progressive impact forces exhibited significant increases in aBMD at the femur after six months. Stone et al. (214) followed women over 12 weeks of either yoga or kick boxing exercise interventions. They suggested that 12 weeks was not enough time to observe significant changes in total body, lumbar spine, or dual femur aBMD; however, increased osteocalcin levels, a bone formation marker, were found. Zribi et al. (215) found that nine weeks of plyometrics performed only two times per week provided a sufficient stimulus to increase total body BMC and BTMs of bone formation but no significant increases in total body aBMD were detected. Lester et al. (6) reported that eight weeks of either progressive aerobic/endurance training, resistance training, or the combination of each program was not enough time to detect significant changes in total body aBMD despite BTM and hormone profiles suggestive of bone formation. The amount of impact loading and the progression of the exercise program are important factors to consider and as previously discussed, perhaps the military exercise intervention in this

study was too short and did not provide enough progression to result in an increase in aBMD in ROTC participants.

Volumetric Bone Mineral Density

No significant changes were found for all 4% and 38% pQCT variables. These findings confirm those of Lester et al. (6) who followed participants during eight week exercise interventions. Participants were divided between four separate groups, control, aerobic or endurance (ET), resistance training (RT), and combined. At the 4% site only the aerobic group demonstrated a significant increase in total vBMD. In the current study, ROTC participants and nearly all control participants reported engaging in both ET and RT, and thus parallels from only the combined group should be drawn. For the 4%, 38%, and 66% pQCT variables in the combined groups, Lester et al. (6) reported no significant changes despite increases in the bone formation markers bone-specific alkaline phosphatase and osteocalcin. Lester and colleagues (6) reported that total impact forces were greatest in running and suggested that perhaps the 4% site is more susceptible to changes due to volume of loading. One additional consideration not addressed by Lester et al. (6), is perhaps the 4% site exhibited significant changes because of the type of bone present. Trabecular bone, which is mainly found at the distal ends of the long bones has a greater surface area to engage in turnover as compared to cortical bone which is primarily found at the 38% and the 66% sites. Potentially, that is why the trabecular rich 4% site was able to exhibit significant changes in such a short period of time as compared to the less metabolically active cortical bone sites.

Baseline pQCT measures in the current study were similar to uninjured military recruits as described by Davey et al. (216) who scanned over 1000 United Kingdom military recruits. They reported the 38% site being the most important for strong correlations to injury risk; however, since this study did not have any reported bone injuries site-specific correlations were not made. Calf mCSA has been reported to be correlated with 38% and 66% SSI and fracture rates in athlete populations (4); however, Davey et al., (216) did not support these findings. In order to investigate the relationship between calf mCSA, load attenuation, and fracture risk, Milgrom and colleagues (136) used a gastrocnemius fatigue model in military recruits. Participants completed a 2-km run and 30-km desert march separately, with gastrocnemius fatigue and tibial compression strain rates being measured post-exercise. Results suggested those with smaller calf mCSA experienced greater fatigue, and were not able to attenuate the load placed on the tibia, which resulted in greater bone strains. Similar findings have been shown in endurance sports, as even early stages of calf fatigue have been shown to increase total bone load (137, 138). Milgrom and colleagues (136) suggested those with greater calf mCSA would be capable of reducing bone loads and potentially reduce the risk of tibial fractures. These results support our findings, and contradict Davey et al., as pre and post 66% mCSA showed strong positive correlations with all tibial bone strength measures. If Milgrom's hypotheses are correct, then tibia fracture risk potentially decreased in both groups as mCSA increased over time.

Physical Performance

Both ROTC and controls were very active, with nearly 85% of all participants meeting the American College of Sports Medicine's physical activity guidelines of at

least 150 min/wk of moderate or at least 75 min/wk of vigorous endurance training (ET), and at least 2 days/wk of resistance training (RT) (217, 218). The inclusion of an activity matched control group is most likely the primary reason for the lack of significant group differences found. It is also possible that controls increased their level of PA despite being encouraged to not make changes to their exercise routines for the duration of the study.

Instead of using common field tests such as number of push ups or sit ups in one minute or a timed two mile run, this study utilized more maximum tests such as grip strength, jump performance, bench press, leg press, and maximal aerobic capacity. This limits the ability to compare performance measures to other large scale studies; however, since both ROTC and controls were physically active most metrics were above average (218). No significant changes occurred in the right hand grip strength; however, left hand grip strength decreased significantly at the post testing period. Despite this change, differences between the left and right hand grip strengths were less than 7% which has been considered to be a significant magnitude of asymmetry (219). Both ROTC and control group grip strength means were considered normal when compared with reference data specifically collected using the Jamar hand dynamometer (220). All jump metrics were within the 95% confidence interval for normative data for young adults (221). Additionally, both ROTC and control bench press:body weight ratios were over 1.1 which is considered good and their leg press:body weight ratios were over 1:2.5 which is considered excellent (218).

Nearly all measures of muscular strength and power increased from pre to midway testing periods for both groups; however, these measures either did not

continue to increase or returned to baseline values by the post testing period. This pattern of change suggests both groups might have been engaging in more resistance training or power movements from the pre to the mid testing period as compared to the mid to the post testing periods. Another possibility is from pre to midway testing performance gains were observed due to adaptations to the training program; however, perhaps due to a lack of training protocol progression, these gains were not sustained from mid to post testing. Without a direct or indirect quantification of training protocols at the midway and post testing periods only speculations can be made regarding the potential changes in training type, time, intensity, volume, and frequency.

ROTC had greater pre and post relative VO_2 peaks as compared to the control group. It should be noted that male and female controls had relative VO_2 peak values categorized as average at the beginning and end the study. ROTC male and female participants exhibited relative VO_2 peak values in good and excellent categories at the beginning and end the study, with four ROTC males exhibiting values over 60 mL/kg/min (218). Evans et al. (35) reported an average 5% increase in estimated aerobic capacity over the 16 week basic training period; the current study demonstrated nearly half of that response observed by Evans. Training intervention length and potential errors in the estimate of aerobic capacity from a two mile run is most likely responsible for this finding.

Limitations

There are several strengths and weakness to consider for this study. The sample size is small compared to previous studies in military personnel, which presented some challenges and unique opportunities. The primary issue with this small population was

the lack of injuries reported, which eliminates the ability to draw inferences about biomarker responses. However, due to the small sample size more precise measures were utilized such as DXA and pQCT as compared to heel ultrasound and skinfold measurements. Additionally, maximum strength and power testing was conducted instead of using more traditional field tests, which minimizes the direct comparison to other large scale studies.

Study duration should also be considered when interpreting these findings. It is important to note that the acute responses of these biomarkers may not be indicative of the long term effects of the exercise on skeletal mass. Studies that reported significant skeletal changes in conjunction with either PTH or sclerostin responses were often 6 or 12 month interventions, and perhaps due to the short duration of the current study, skeletal and biomarker changes were too small to detect.

Many questionnaires were used at the beginning of the study to assess health status and eligibility, including calcium intake and reported auxiliary physical activity questionnaires. It would have been helpful to collect this information at the pre, midway, and post testing periods, which would allow for the description of potential changes in PA or calcium intake over time.

Lastly, ROTC members are not yet commissioned military officers, instead they are college students who are preparing for a career in the military. The findings of this study describe how ROTC programs may prepare these students for future enlistment but these findings should not be generalized to other types of basic training where the frequency, intensity, and duration of training may surpass this eight week intervention.

Chapter 5: Conclusions

The purpose of this study was to determine the effects of an eight week military training intervention on PTH and sclerostin serum concentrations, and aBMD of the total body, dual femur, and lumbar spine, and bone geometry of the tibia, in healthy, college-aged USMC and Naval Reserve Officers' Training Corps (ROTC) members compared to a matched control group. A secondary purpose was to determine the relationship between PTH and sclerostin and bone variables. Lastly, group differences in body composition, muscular strength and power measures, and aerobic fitness and their relationships to PTH and sclerostin were examined.

Research Questions

1. Will an eight week military training intervention period (MTIP) significantly alter PTH and sclerostin serum concentrations, and will these responses be different from those in a matched control group who does not participate in the MTIP?

   No significant group × time interactions, or main effects for time or group were found for either PTH or sclerostin. It was hypothesized that PTH would increase while sclerostin would decrease in ROTC but not controls. The direction of biomarker responses was expected, but the magnitude of changes did not reach statistical significance for either group.

2. Will an eight week MTIP result in significant total body aBMD changes and site specific aBMD changes at the lumbar spine and dual femur, and will these changes be different from those in a matched control group who does not participate in the MTIP?

Significant group × time interactions were found as dominant femoral neck aBMD significantly increased in ROTC and decreased in controls. The dominant total hip aBMD significantly increased in ROTC; while, dominant total hip BMC significantly increased in controls. It should be noted that none of these statistically significant changes in ROTC or controls exceeded the CV% for the DXA enough to be considered to be clinically significant.

3. Will an eight week MTIP alter bone content, geometry, and strength of the 4%, 38%, 66% non-dominant tibia sites, and will these changes be different from those in a matched control group who does not participate in the MTIP?
   No significant group × time interactions, or main effects for time or group were found for most of the pQCT variables. A significant time effect was observed as 66% total BMC and mCSA both increased from pre to post testing periods. Due to the lack of interactions observed, hypotheses regarding pQCT variables were not confirmed.

Sub Questions

1. Will there be significant relationships between serum PTH and sclerostin and total body and site-specific aBMD, BFLBM and fat mass?
   PTH showed few significant correlations with measures of aBMD and body composition variables for both groups; however, sclerostin concentrations in ROTC participants were significantly correlated with many measures of aBMD, and BFLBM.

2. Will there be significant relationships between serum PTH and sclerostin and 4%, 38%, 66% non-dominant tibia sites measures of bone strength and geometry?

   PTH was significantly associated with few pQCT variables. Sclerostin concentrations in ROTC participants demonstrated strong positive associations with pQCT variables, especially indices of bone strength at the 4%, 38%, and 66% tibia sites. Sclerostin was also a significant predictor of bone strength at the 4%, 38%, and 66% tibia sites confirming the proposed hypothesis

3. Will there be significant relationships between serum PTH and sclerostin and muscular power and strength?

   PTH was not significantly correlated with any muscular power or strength measures for either group. Sclerostin was significantly correlated with hand grip strength, bench press, leg press, and jump power at all testing points. In general, the relationship between sclerostin and bone variables was stronger and incorporated a greater proportion of measures as compared to muscular strength and power performance tests.

4. Will there be significant relationships between serum PTH and sclerostin and aerobic capacity?

   Sclerostin was significantly correlated with absolute VO_2 peak at both testing points in ROTC only; while PTH was not consistently significantly associated with any variables. Much like muscle performance testing, the relationship between sclerostin and bone variables was stronger and incorporated a greater proportion of measures as compared to aerobic capacity testing.

Clinical Significance

Since no bone injuries were reported, associations between the biomarkers, PTH and sclerostin, cannot be made within this cohort; however, the positive correlations between sclerostin and measures of bone strength could still provide important information. Since distal tibial fractures are among the most common in military cohorts and Davey et al. (216) report the 38% site being most important for injury risk it is encouraging to report that %Δ in 38% SSI was best predicted by %Δ sclerostin and 66% mCSA as compared to other common field measures of BF% or performance measures.

Control participants exhibited a statistically significant 1.02% decrease in dominant femoral neck aBMD which was close to the CV% of the DXA scanner. Although this change is rather small it could be argued that due to the age of the study population, any increase or decrease in aBMD is clinically important, as peak bone mass has not yet been reached. Peak aBMD is a significant predictor of fracture risk later in life (222-224); however, studies using pharmacological interventions report changes as small as 1-2% in aBMD can result in significant fracture reduction (225-227). It should be noted that many studies utilizing DXA do not report CV% values and thus their findings should be considered with caution.

Hinton et al. (42) demonstrated, across a 12 month intervention, an average of 4.5% reduction in sclerostin in the resistance trained group which coincided with a 1.68% gain in lumbar spine aBMD, and 0.88% gain in total hip aBMD. ROTC participants exhibited a 4.8% reduction in serum sclerostin concentrations, which corresponded with a 0.52% gain in the lumbar spine aBMD and a 0.64% gain in total

hip aBMD; however, these changes were not significant. Armamentro-Villareal et al. (228), found after a 12 month weight loss study serum sclerostin increased by nearly 10% which translated to a 2-3% loss in hip aBMD. Few longitudinal studies have been conducted with sclerostin and many more need to be published before clinical recommendations for significant percent changes can be made.

Recommendations for Future Research

The correlations and predictive power of sclerostin may provide additional information for future studies investigating this population. Interested investigators are encouraged to alter the current research design by lengthening the duration of the intervention, increasing the sample size, and increasing the frequency of blood sampling.

Due to the recruitment of a physically active control group the general effects of exercise as a confounding variable were minimized. This reduced the number of group differences observed; however, it also strengthened the integrity of interactions reported. Currently, very little is published on sclerostin responses to longitudinal exercise interventions; however, the group specific associations between sclerostin and skeletal measures in ROTC is very interesting and deserving of further investigation.

Lastly, maximum strength and power exercises have slowly become integrated into updated military training programs (50, 190, 192). All branches of the military are starting to swap out long distance runs and sit ups for explosive, maximal effort power movements and future studies using this population should aim to reflect this transition with more testing of power and strength measures such as aerobic capacity, bench press, and leg press (192).

References

1. Sims NA, Martin TJ. Coupling the activities of bone formation and resorption: a multitude of signals within the basic multicellular unit. *BoneKey reports*.2014;3.

2. Beck BR, Snow CM. Bone health across the lifespan - Exercising our options. *Exercise and Sport Sciences Reviews*. 2003;31(3):117-22.

3. Yanovich R, Heled Y, Hughes J. The Biomechanical Basis for Increased Risk of Overuse Musculoskeletal Injuries in Female Soldiers. In. *The Mechanobiology and Mechanophysiology of Military-Related Injuries*: Springer; 2015, pp. 187-206.

4. Bennell, Brukner P. Preventing and managing stress fractures in athletes. *Physical Therapy in Sport*. 2005;6(4):171-80.

5. Cauley JA. Osteoporosis: fracture epidemiology update 2016. *Current Opinion in Rheumatology*. 2017;29(2):150-6.

6. Lester ME, Urso ML, Evans RK et al. Influence of exercise mode and osteogenic index on bone biomarker responses during short-term physical training. *Bone*. 2009;45(4):768-76.

7. Scofield DE, Kardouni JR. The Tactical Athlete: A Product of 21st Century Strength and Conditioning. *Strength & Conditioning Journal*. 2015;37(4):2-7.

8. Armstrong DW, Rue J-PH, Wilckens JH, Frassica FJ. Stress fracture injury in young military men and women. *Bone*. 2004;35(3):806-16.

9. Bell NS, Mangione TW, Hemenway D, Amoroso PJ, Jones BH. High injury rates among female army trainees: a function of gender? *American Journal of Preventive Medicine*. 2000;18(3):141-6.

10. Cosman F, Ruffing J, Zion M et al. Determinants of stress fracture risk in United States Military Academy cadets. *Bone*. 2013;55(2):359-66.

11. Hauschild V, Roy T, Grier T, Schuh A, Jones BH. Foot Marching, Load Carriage, and Injury Risk. Army Public Health Center Aberdeen Proving Ground-Edgewood Area United States. Available from: *Army Public Health* 2016.

12. Council NR. Assessing fitness for military enlistment: Physical, medical, and mental health standards. *National Academies Press;* 2006.

13. Wood AM, Hales R, Keenan A et al. Incidence and time to return to training for stress fractures during military basic training. *Journal of Sports Medicine*. 2014.

14. Biberdorf C, Evans R. Army lab tackles problem of military stress fractures. *Warrior Magazine at the US Army Soldier Systems Center.* Special to American Forces Press Service NATICK, Mass. May. 2004;19.

15. Giladi M, Milgrom C, Simkin A et al. Stress fractures and tibial bone width. A risk factor. *Bone & Joint Journal*. 1987;69(2):326-9.

16. Milgrom. The Israeli elite infantry recruit: a model for understanding the biomechanics of stress fractures. *Journal of the Royal College of Surgeons of Edinburgh*. 1988;34(6 Suppl):S18-22.

17. Välimäki V-V, Alfthan H, Lehmuskallio E et al. Risk factors for clinical stress fractures in male military recruits: a prospective cohort study. *Bone*. 2005;37(2): 267-73.

18. Lacroix H, Keeman J. An unusual stress fracture of the fibula in a long-distance runner. *Archives of Orthopaedic and Trauma Surgery*. 1992;111(5):289-90.

19. Beck, Ruff C, Shaffer R, Betsinger K, Trone D, Brodine S. Stress fracture in military recruits: gender differences in muscle and bone susceptibility factors. *Bone*. 2000;27(3):437-44.

20. Johansson H, Odén A, Kanis JA et al. A meta-analysis of reference markers of bone turnover for prediction of fracture. *Calcified Tissue International*. 2014; 94(5):560-7.

21. Seibel MJ. Biochemical markers of bone turnover part I: biochemistry and variability. *The Clinical Biochemist. Reviews/Australian Association of Clinical Biochemists.* 2005;26(4):97.

22. Wheater G, Elshahaly M, Tuck SP, Datta HK, van Laar JM. The clinical utility of bone marker measurements in osteoporosis. *Journal of Translational Medicine*. 2013;11(1):201.

23. Haarhaus M, Monier-Faugere M-C, Magnusson P, Malluche HH. Bone alkaline phosphatase isoforms in hemodialysis patients with low versus non-low bone turnover: a diagnostic test study. *American Journal of Kidney Diseases*. 2015; 66(1):99-105.

24. Rosen H, Moses A, Garber J et al. Serum CTX: a new marker of bone resorption that shows treatment effect more often than other markers because of low coefficient of variability and large changes with bisphosphonate therapy. *Calcified Tissue International*. 2000;66(2):100-3.

25. Rissanen JP, Suominen MI, Peng Z, Halleen JM. Secreted tartrate-resistant acid phosphatase 5b is a Marker of osteoclast number in human osteoclast cultures and the rat ovariectomy model. *Calcified Tissue International*. 2008;82(2):108-15.

26. Bonnick SL, Shulman L. Monitoring osteoporosis therapy: bone mineral density, bone turnover markers, or both? *The American Journal of Medicine*. 2006;119(4):S25-S31.

27. Eastell R, Barton I, Hannon R, Chines A, Garnero P, Delmas P. Relationship of early changes in bone resorption to the reduction in fracture risk with risedronate. *Journal of Bone and Mineral Research*. 2003;18(6):1051-6.

28. Bennell, Malcolm SA, Thomas SA et al. Risk factors for stress fractures in track and field athletes A twelve-month prospective study. *The American Journal of Sports Medicine*. 1996;24(6):810-8.

29. Bennell K, Malcolm S, Brukner P et al. A 12-month prospective study of the relationship between stress fractures and bone turnover in athletes. *Calcified Tissue International*. 1998;63(1):80-5.

30. Scott JP, Greeves JP, Casey A, Sale C, Dutton J, Fraser WD. Investigations into Changes in Bone Turnover with Acute, Weight-Bearing Exercise in Healthy, Young Men. *Military Proceedings*, UK; 2009.

31. Moran DS, Finestone AS, Arbel Y, Shabshin N, Laor A. A simplified model to predict stress fracture in young elite combat recruits. *The Journal of Strength & Conditioning Research*. 2012;26(9):2585-92.

32. Bouassida A, Latiri I, Bouassida S et al. Parathyroid hormone and physical exercise: a brief review. *Journal of Sports Science & Medicine*. 2006;5(3):367.

33. Cavalier E, Plebani M, Delanaye P, Souberbielle J-C. Considerations in parathyroid hormone testing. *Clinical Chemistry and Laboratory Medicine*. 2015;53(12):1913-9.

34. Andersen NE, Karl JP, Cable SJ et al. Vitamin D status in female military personnel during combat training. *Journal of the International Society of Sports Nutrition*. 2010;7:38-.

35. Evans RK, Antczak AJ, Lester M, Yanovich R, Israeli E, Moran DS. Effects of a 4-month recruit training program on markers of bone metabolism. *Medicine & Science in Sports & Exercise*. 2008;40(11):S660-S70.

36. Van Bezooijen RL, Svensson JP, Eefting D et al. Wnt but not BMP signaling is involved in the inhibitory action of sclerostin on BMP-stimulated bone formation. *Journal of Bone and Mineral Research*. 2007;22(1):19-28.

37. Dallas SL, Bonewald LF. Dynamics of the transition from osteoblast to osteocyte. *Annals of the New York Academy of Sciences*. 2010;1192(1):437-43.

38. Falk B, Haddad F, Klentrou P et al. Differential sclerostin and parathyroid hormone response to exercise in boys and men. *Osteoporosis International*. 2016;27(3):1245-9.

39. Robling AG, Niziolek PJ, Baldridge LA et al. Mechanical stimulation of bone in vivo reduces osteocyte expression of Sost/sclerostin. *Journal of Biological Chemistry*. 2008;283(9):5866-75.

40. Mezil YA, Allison D, Kish K et al. Response of bone turnover markers and cytokines to high-intensity low-impact exercise. *Medicine and Science in Sports and Exerc*ise. 2015;47(7):1495-502.

41. Kerschan-Schindl K, Thalmann MM, Weiss E et al. Changes in serum levels of myokines and Wnt-antagonists after an ultramarathon race. *PloS one*. 2015;10(7):e0132478.

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

43. Ardawi MSM, Rouzi AA, Al-Sibiani SA, Al-Senani NS, Qari MH, Mousa SA. High serum sclerostin predicts the occurrence of osteoporotic fractures in postmenopausal women: the Center of Excellence for Osteoporosis Research Study. *Journal of Bone and Mineral Research*. 2012;27(12):2592-602.

44. Ardawi M-SM, Akhbar DH, AlShaikh A et al. Increased serum sclerostin and decreased serum IGF-1 are associated with vertebral fractures among postmenopausal women with type-2 diabetes. *Bone*. 2013;56(2):355-62.

45. Leslie WD, Orwoll ES, Nielson CM et al. Estimated lean mass and fat mass differentially affect femoral bone density and strength index but are not FRAX independent risk factors for fracture. *Journal of Bone and Mineral Research*. 2014;29(11):2511-9.

46. Øyen J, Gjesdal CG, Nygård OK et al. Smoking and body fat mass in relation to bone mineral density and hip fracture: the Hordaland Health Study. *PloS one*. 2014;9(3):e92882.

47. Yang S, Center J, Eisman J, Nguyen T. Association between fat mass, lean mass, and bone loss: the Dubbo osteoporosis epidemiology study. *Osteoporosis International.* 2015;26(4):1381-6.

48. Shaffer RA, Rauh MJ, Brodine SK, Trone DW, Macera CA. Predictors of stress fracture susceptibility in young female recruits. *The American Journal of Sports Medicine*. 2006;34(1):108-15.

49. Malkov S, Cawthon PM, Peters KW et al. Hip Fractures Risk in Older Men and Women Associated With DXA-Derived Measures of Thigh Subcutaneous Fat Thickness, Cross-Sectional Muscle Area, and Muscle Density. *Journal of Bone and Mineral Research*. 2015;30(8):1414-21.

50. Nindl BC. Physical training strategies for military women's performance optimization in combat-centric occupations. *The Journal of Strength & Conditioning Research*. 2015;29:S101-S6.

51. Lee D. Stress fractures, active component, US Armed Forces, 2004-2010. *Medical Surveillance Monthly Report*. 2011;18(5):8-11.

52. Hauret KG. The physical training and rehabilitation program: duration of rehabilitation and final outcome of injuries in basic combat training. *Military Medicine*. 2001;166(9):820.

53. Reis JP, Trone DW, Macera CA, Rauh MJ. Factors associated with discharge during marine corps basic training. *Military Medicine*. 2007;172(9):936-41.

54. Langton CM, Njeh CF. *The Physical Measurement of Bone*. CRC Press; 2016.

55. Heyward VH, Wagner DR. *Applied Body Composition Assessment*. Human Kinetics; 2004.

56. Rauch F, Schoenau E. Peripheral quantitative computed tomography of the proximal radius in young subjects—new reference data and interpretation of results. *Journal of Musculoskelet Neuronal Interact*. 2008;8(3):217-26.

57. Goodman HM. *Basic Medical Endocrinology*. Academic Press; 2010.

58. Cointry G, Ferretti JL, Reina P, Nocciolono L, Rittweger J, Capozza R. 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. *Journal of Musculoskeletal and Neuronal Interactions*. 2014;14(1):29-40.

59. Rosen CJ, Compston JE, Lian JB. *ASBMR Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*. John Wiley & Sons; 2009.

60. VanPutte C. *Seeley's Anatomy & Physiology*. McGraw-Hill Higher Education; 2016.

61. Swinford RR, Warden SJ. Factors affecting short-term precision of musculoskeletal measures using peripheral quantitative computed tomography (pQCT). *Osteoporosis International*. 2010;21(11):1863-70.

62. Schousboe JT, Shepherd JA, Bilezikian JP, Baim S. Executive summary of the 2013 international society for clinical densitometry position development conference on bone densitometry. *Journal of Clinical Densitometry*. 2013; 16(4):455-66.

63. Bieglmayer C, Prager G, Niederle B. Kinetic analyses of parathyroid hormone clearance as measured by three rapid immunoassays during parathyroidectomy. *Clinical Chemistry*. 2002;48(10):1731-8.

64. Kim S, Bemben MG, Knehans AW, Bemben DA. Effects of an 8-month Ashtanga-based yoga intervention on bone metabolism in middle-aged premenopausal women: a randomized controlled study. *Journal of Sports Science & Medicine*. 2015;14(4):756.

65. Carey JJ, Delaney MF. T-scores and Z-scores. *Clinical Reviews in Bone and Mineral Metabolism*. 2010;8(3):113-21.

66. Sapir-Koren R, Livshits G. Osteocyte control of bone remodeling: is sclerostin a key molecular coordinator of the balanced bone resorption–formation cycles? *Osteoporosis International*. 2014;25(12):2685-700.

67. van Lierop AH, Hamdy NA, van Egmond ME, Bakker E, Dikkers FG, Papapoulos SE. Van Buchem disease: clinical, biochemical, and densitometric features of patients and disease carriers. *Journal of Bone and Mineral Research*. 2013;28(4):848-54.

68. Lewiecki EM. Management of osteoporosis. *Clinical and Molecular Allergy*. 2004;2(1):9.

69. Frost HM. Muscle, bone, and the Utah paradigm: a 1999 overview. *Medicine & Science in Sports & Exercise*. 2000;32(5):911-7.

70. Skerry TM. One mechanostat or many? Modifications of the site-specific response of bone to mechanical loading by nature and nurture. *Journal of Musculoskelet Neuronal Interactions*. 2006;6(2):122-7.

71. Frost HM. Bone “mass” and the “mechanostat”: a proposal. *The Anatomical Record*. 1987;219(1):1-9.

72. Turner CH, Pavalko FM. Mechanotransduction and functional response of the skeleton to physical stress: the mechanisms and mechanics of bone adaptation. *Journal of Orthopdedic Science*. 1998;3(6):346-55.

73. Bonewald. Osteocytes as dynamic multifunctional cells. *Annals of the New York Academy of Sciences.* 2007;1116:281-90.

74. Franz-Odendaal TA, Hall BK, Witten PE. Buried alive: how osteoblasts become osteocytes. *Developmental Dynamics*. 2006;235(1):176-90.

75. Tanaka K, Yamaguchi Y, Hakeda Y. Isolated chick osteocytes stimulate formation and bone-resorbing activity of osteoclast-like cells. *Journal of Bone and Mineral Metabolism*. 1995;13(2):61-70.

76. Bonewald. The amazing osteocyte. *Journal of Bone and Mineral Research*. 2011;26(2):229-38.

77. Lane NE, Yao W, Balooch M et al. Glucocorticoid-Treated Mice Have Localized Changes in Trabecular Bone Material Properties and Osteocyte Lacunar Size That Are Not Observed in Placebo-Treated or Estrogen-Deficient Mice. *Journal of Bone and Mineral Research*. 2006;21(3):466-76.

78. Baud C, Dupont D. The fine structure of the osteocytes in the adult compact bone. *Electron Microscopy*. 1962;2.

79. Otto F, Thornell AP, Crompton T et al. Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell*. 1997;89(5):765-71.

80. Nakashima K, Zhou X, Kunkel G et al. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell*. 2002;108(1):17-29.

81. Lee K-S, Kim H-J, Li Q-L et al. Runx2 is a common target of transforming growth factor β1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2C12. *Molecular and Cellular Biology*. 2000; 20(23):8783-92.

82. Ross FP. Osteoclast biology and bone resorption. *Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism*. 2006;6:30-5.

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

84. Stenbeck G, Horton MA. Endocytic trafficking in actively resorbing osteoclasts. *Journal of Cell Science*. 2004;117(6):827-36.

85. Peacock M, Turner CH, Econs MJ, Foroud T. Genetics of osteoporosis. *Endocrine Reviews*. 2002;23(3):303-26.

86. Wang, Cheng S, Alén M, Seeman E. Bone's structural diversity in adult females is established before puberty. *The Journal of Clinical Endocrinology & Metabolism*. 2009;94(5):1555-61.

87. Bjørnerem Å, Johnsen SL, Nguyen TV, Kiserud T, Seeman E. The shifting trajectory of growth in femur length during gestation. *Journal of Bone and Mineral Research*. 2010;25(5):1029-33.

88. Trinkaus E, Churchill SE, Ruff CB. Postcranial robusticity in Homo. II: Humeral bilateral asymmetry and bone plasticity. *American Journal of Physical Anthropology*. 1994;93(1):1-34.

89. Ruff CB, Walker A, Trinkaus E. Postcranial robusticity in Homo. III: ontogeny. *American Journal of Physical Anthropology*. 1994;93(1):35-54.

90. Hui S, Slemenda C, Johnston C. The contribution of bone loss to postmenopausal osteoporosis. *Osteoporosis International*. 1990;1(1):30-4.

91. Hendrickx G, Boudin E, Van Hul W. A look behind the scenes: the risk and pathogenesis of primary osteoporosis. *Nature Reviews Rheumatology*. 2015;11(8):462-74.

92. Nojiri H, Saita Y, Morikawa D et al. Cytoplasmic superoxide causes bone fragility owing to low-turnover osteoporosis and impaired collagen cross-linking. *Journal of Bone and Mineral Research*. 2011;26(11):2682-94.

93. Almeida M. Unraveling the role of FoxOs in bone—insights from mouse models. *Bone*. 2011;49(3):319-27.

94. Jilka RL, O'Brien CA, Roberson PK, Bonewald LF, Weinstein RS, Manolagas SC. Dysapoptosis of osteoblasts and osteocytes increases cancellous bone formation but exaggerates cortical porosity with age. *Journal of Bone and Mineral Research*. 2014;29(1):103-17.

95. YU S-h, HONG A. Runx2 promotes osteogenic differentiating C2C12 cells through inhibiting macroautophagy. *Chinese Journal of Pathophysiol*. 2013; 29(3):481-7.

96. Devlin MJ. Estrogen, exercise, and the skeleton. *Evolutionary Anthropology: Issues, News, and Reviews*. 2011;20(2):54-61.

97. Melville KM, Kelly NH, Surita G et al. Effects of Deletion of ERα in Osteoblast-Lineage Cells on Bone Mass and Adaptation to Mechanical Loading Differ in Female and Male Mice. *Journal of Bone and Mineral Research*. 2015;30(8):1468-80.

98. Neu C, Rauch F, Manz F, Schoenau E. Modeling of cross-sectional bone size, mass and geometry at the proximal radius: a study of normal bone development using peripheral quantitative computed tomography. *Osteoporosis International.* 2001;12(7):538-47.

99. Havill LM, Mahaney MC, L Binkley T, L Specker B. Effects of genes, sex, age, and activity on BMC, bone size, and areal and volumetric BMD. *Journal of Bone and Mineral Research*. 2007;22(5):737-46.

100. Akhter M, Lappe J, Davies K, Recker R. Transmenopausal changes in the trabecular bone structure. *Bone*. 2007;41(1):111-6.

101. Manolagas SC. From estrogen-centric to aging and oxidative stress: a revised perspective of the pathogenesis of osteoporosis. *Endocrine Reviews*. 2010;31(3):266-300.

102. Torgerson DJ, Bell-Syer SE. Hormone replacement therapy and prevention of nonvertebral fractures: a meta-analysis of randomized trials. *Journal of American Medical Association*. 2001;285(22):2891-7.

103. Riggs BL, Melton LJ, Robb RA et al. A population-based assessment of rates of bone loss at multiple skeletal sites: evidence for substantial trabecular bone loss in young adult women and men. *Journal of Bone and Mineral Research*. 2008;23(2):205-14.

104. Riggs BL, Khosla S, Melton LJ. A unitary model for involutional osteoporosis: estrogen deficiency causes both type I and type II osteoporosis in postmenopausal women and contributes to bone loss in aging men. *Journal of Bone and Mineral Research*. 1998;13(5):763-73.

105. Khosla S, Melton III LJ, Atkinson EJ, O'fallon W, Klee GG, Riggs BL. Relationship of serum sex steroid levels and bone turnover markers with bone mineral density in men and women: a key role for bioavailable estrogen 1. *The Journal of Clinical Endocrinology & Metabolism*. 1998;83(7):2266-74.

106. Raisz LG. Pathogenesis of osteoporosis: concepts, conflicts, and prospects. *The Journal of Clinical Investigation*. 2005;115(12):3318-25.

107. Nair KS, Rizza RA, O'brien P et al. DHEA in elderly women and DHEA or testosterone in elderly men. *New England Journal of Medicine.* 2006; 355(16):1647-59.

108. Vincent A, Riggs BL, Atkinson EJ, Oberg AL, Khosla S. Effect of estrogen replacement therapy on parathyroid hormone secretion in elderly postmenopausal women. *Menopause*. 2003;10(2):165-71.

109. Khosla S, Riggs BL. Pathophysiology of age-related bone loss and osteoporosis. *Endocrinology and Metabolism Clinics*. 2005;34(4):1015-30.

110. Bischoff-Ferrari HA, Kiel DP, Dawson-Hughes B et al. Dietary Calcium and Serum 25-Hydroxyvitamin D Status in Relation to BMD Among US Adults. *Journal of Bone and Mineral Research*. 2009;24(5):935-42.

111. Pu F, Chen N, Xue S. Calcium intake, calcium homeostasis and health. *Food Science and Human Wellness*. 2016;5(1):8-16.

112. Brownie S. Nutritional wellbeing for older people. *Journal of the Australian Traditional-Medicine Society*. 2013;19(3):140.

113. Delgado-Calle J, Tu X, Pacheco-Costa R et al. Control of bone anabolism in response to mechanical loading and PTH by distinct mechanisms downstream of the PTH receptor. *Journal of Bone and Mineral Research*. 2017;32(3):522-35.

114. MacLaughlin J, Holick MF. Aging decreases the capacity of human skin to produce vitamin D3. *Journal of Clinical Investigation*. 1985;76(4):1536.

115. Shea B, Wells G, Cranney A et al. VII. Meta-analysis of calcium supplementation for the prevention of postmenopausal osteoporosis. *Endocrine Reviews*. 2002;23(4):552-9.

116. Bischoff-Ferrari HA, Dietrich T, Orav EJ et al. Higher 25-hydroxyvitamin D concentrations are associated with better lower-extremity function in both active and inactive persons aged≥ 60 y. *The American Journal of Clinical Nutrition*. 2004;80(3):752-8.

117. Wicherts IS, van Schoor NM, Boeke AJP et al. Vitamin D status predicts physical performance and its decline in older persons. *The Journal of Clinical Endocrinology & Metabolism*. 2007;92(6):2058-65.

118. Whiting S, Kohrt W, Warren M, Kraenzlin M, Bonjour J-P. Food fortification for bone health in adulthood: a scoping review. *European Journal of Clinical Nutrition*. 2016;70(10):1099.

119. Shen C-L, von Bergen V, Chyu M-C et al. Fruits and dietary phytochemicals in bone protection. *Nutrition Research*. 2012;32(12):897-910.

120. Heaney RP, Layman DK. Amount and type of protein influences bone health. *The American Journal of Clinical Nutrition*. 2008;87(5):1567S-70S.

121. Ronsen O, Sundgot-Borgen J, Maehlum S. Supplement use and nutritional habits in Norwegian elite athletes. *Scandinavian Journal of Medicine & Science in Sports*. 1999;9(1):28-35.

122. Zanker CL, Swaine IL. Responses of bone turnover markers to repeated endurance running in humans under conditions of energy balance or energy restriction. *European Journal of Applied Physiology*. 2000;83(4-5):434-40.

123. Ihle R, Loucks AB. Dose-response relationships between energy availability and bone turnover in young exercising women. *Journal of Bone and Mineral Research*. 2004;19(8):1231-40.

124. Mountjoy M, Sundgot-Borgen J, Burke L et al. The IOC consensus statement: beyond the Female Athlete Triad--Relative Energy Deficiency in Sport (RED-S). *British Journal of Sports Medicine.* 2014;48(7):491-7.

125. Maurel D, Boisseau N, Benhamou C, Jaffre C. Alcohol and bone: review of dose effects and mechanisms. *Osteoporosis International*. 2012;23(1):1-16.

126. Kanis JA, Johnell O, Odén A et al. Smoking and fracture risk: a meta-analysis. *Osteoporosis International*. 2005;16(2):155-62.

127. Yoon V, Maalouf N, Sakhaee K. The effects of smoking on bone metabolism. *Osteoporosis International*. 2012;23(8):2081-92.

128. Duncan RL, Turner CH. Mechanotransduction and the Functional-Response of Bone to Mechanical Strain. *Calcified Tissue International*. 1995;57(5):344-58.

129. Rubin CT, Lanyon LE. Regulation of Bone Mass by Mechanical Strain Magnitude. *Calcified Tissue International*. 1985;37(4):411-7.

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

131. Frost HM, Ferretti J, Jee W. Perspectives: some roles of mechanical usage, muscle strength, and the mechanostat in skeletal physiology, disease, and research. *Calcified Tissue International*. 1998;62(1):1-7.

132. Kiratli BJ. Immobilization Osteoporosis. In. *Atlas of Osteoporosis*: Springer; 2003, pp. 149-64.

133. Martin, Burr DB, Sharkey NA. *Skeletal Tissue Mechanics*. Springer; 1998.

134. Scott SH, Winter DA. Internal forces of chronic running injury sites. *Medicine & Science in Sports & Exercise*. 1990;22(3):357-69.

135. Burr. 7 Bone, Exercise, and Stress Fractures. *Exercise and Sport Sciences Reviews*. 1997;25(1):171-94.

136. Milgrom, Radeva-Petrova DR, Finestone A et al. The effect of muscle fatigue on in vivo tibial strains. *Journal of Biomechanics*. 2007;40(4):845-50.

137. Fyhrie DP, Milgrom C, Hoshaw S et al. Effect of fatiguing exercise on longitudinal bone strain as related to stress fracture in humans. *Annals of Biomedical Engineering*. 1998;26(4):660-5.

138. Yoshikawa T, Mori S, Santiesteban A et al. The effects of muscle fatigue on bone strain. *Journal of Experimental Biology*. 1994;188(1):217-33.

139. Friberg O. Leg length asymmetry in stress fractures. A clinical and radiological study. *The Journal of Sports Medicine and Physical Fitness*. 1982;22(4):485-8.

140. Brunet ME, Cook SD, Brinker M, Dickinson J. A survey of running injuries in 1505 competitive and recreational runners. *The Journal of Sports Medicine and Physical Fitness*. 1990;30(3):307-15.

141. Korpelainen R, Orava S, Karpakka J, Siira P, Hulkko A. Risk Factors for Recurrent Stress Fractures in Athletes. *The American Journal of Sports Medicine*. 2001;29(3):304-10.

142. Andrish JT, Bergfeld JA, Walheim J. A prospective study on the management of shin splints. *Journal of Bone Joint Surgery-American*. 1974;56(8):1697-700.

143. Gardner Jr LI, Dziados JE, Jones BH et al. Prevention of lower extremity stress fractures: a controlled trial of a shock absorbent insole. *American Journal of Public Health*. 1988;78(12):1563-7.

144. Milgrom, Finestone A, Shlamkovitch N et al. Prevention of overuse injuries of the foot by improved shoe shock attenuation: a randomized prospective study. *Clinical Orthopaedics and Related Research*. 1992;281:189-92.

145. Milgrom, Giladi M, Kashtan H et al. A prospective study of the effect of a shock-absorbing orthotic device on the incidence of stress fractures in military recruits. *Foot & Ankle*. 1985;6(2):101-4.

146. Giladi M, Milgrom C, Danon Y, Aharonson Z. The correlation between cumulative march training and stress fractures in soldiers. *Military Medicine*. 1985;150(11):600.

147. Schwellnus MP, Jordaan G, Noakes TD. Prevention of common overuse injuries by the use of shock absorbing insoles. A prospective study. *American Journal of Sports Medicine*. 1990;18(6):636-41.

148. Smith W, Walter Jr J, Bailey M. Effects of insoles in coast guard basic training footwear. *Journal of the American Podiatric Medical Association*. 1985;75(12):644-7.

149. Rome K, Handoll HH, Ashford RL. Interventions for preventing and treating stress fractures and stress reactions of bone of the lower limbs in young adults. *The Cochrane Library*. 2005.

150. Ahlborg HG, Johnell O, Turner CH, Rannevik G, Karlsson MK. Bone loss and bone size after menopause. *New England Journal of Medicine*. 2003;349(4):327-34.

151. Burr, Milgrom C, Boyd RD, Higgins WL, Robin G, Radin EL. Experimental stress fractures of the tibia. Biological and mechanical aetiology in rabbits. *Journal of Bone and Joint Surgery of Britian*. 1990;72(3):370-5.

152. Matheson GO, Clement DB, McKenzie DC, Taunton JE, Lloyd-Smith DR, MacIntyre JG. Stress fractures in athletes. A study of 320 cases. *American Journal of Sports Medicine*. 1987;15(1):46-58.

153. Milgrom, Gildadi M, Simkin A et al. The area moment of inertia of the tibia: a risk factor for stress fractures. *Journal of Biomechanics*. 1989;22(11-12):1243-8.

154. Dennis J. A new system of measurement in X-ray work. *Dental Cosmos*. 1897;39:445-54.

155. Kelly T, Berger N, Richardson T. DXA body composition: theory and practice. *Applied Radiation and Isotopes*. 1998;49(5-6):511-3.

156. Bouxsein. Technology insight: noninvasive assessment of bone strength in osteoporosis. *Nature Clinical Practice Rheumatology*. 2008;4(6):310-8.

157. Lewiecki EM, Binkley N. DXA: 30years and counting introduction to the 30th anniversary issue. *Bone*. 2016.

158. McCloskey EV, Odén A, Harvey NC et al. Adjusting fracture probability by trabecular bone score. *Calcified Tissue International*. 2015;96(6):500-9.

159. Beck. Extending DXA beyond bone mineral density: understanding hip structure analysis. *Current Osteoporosis Reports*. 2007;5(2):49-55.

160. Golestani R, Tio RA, Zeebregts CJ et al. Abdominal aortic calcification detected by dual X-ray absorptiometry: a strong predictor for cardiovascular events. *Annals of Medicine*. 2010;42(7):539-45.

161. Albanese CV, Diessel E, Genant HK. Clinical applications of body composition measurements using DXA. *Journal of Clinical Densitometry*. 2003;6(2):75-85.

162. Ohnaru K, Sone T, Tanaka K et al. Hip structural analysis: a comparison of DXA with CT in postmenopausal Japanese women. *Springerplus*. 2013;2:331.

163. Riggs BL, Melton LJ, Robb RA et al. Population-based study of age and sex differences in bone volumetric density, size, geometry, and structure at different skeletal sites. *Journal of Bone and Mineral Research*. 2004;19(12):1945-54.

164. Martin DE, Severns AE, Kabo JM. Determination of mechanical stiffness of bone by pQCT measurements: correlation with non-destructive mechanical four-point bending test data. *Journal of Biomechanics*. 2004;37(8):1289-93.

165. Genant HK, Boyd D. Quantitative bone mineral analysis using dual energy computed tomography. *Investigative Radiology*. 1977;12(6):545-51.

166. Muller A, Ruegsegger E, Ruegsegger P. Peripheral QCT: a low-risk procedure to identify women predisposed to osteoporosis. *Physics in Medicine and Biology*. 1989;34(6):741.

167. Wegrzyn J, Roux JP, Arlot ME et al. Role of trabecular microarchitecture and its heterogeneity parameters in the mechanical behavior of ex vivo human L3 vertebrae. *Journal of Bone and Mineral Research*. 2010;25(11):2324-31.

168. Vasikaran S, Eastell R, Bruyère O et al. Markers of bone turnover for the prediction of fracture risk and monitoring of osteoporosis treatment: a need for international reference standards. *Osteoporosis International*. 2011;22(2):391-420.

169. Nakamura, Dohi Y, Akahane M et al. Osteocalcin secretion as an early marker of in vitro osteogenic differentiation of rat mesenchymal stem cells. *Tissue Engineering* Part C: Methods. 2009;15(2):169-80.

170. Halleen JM, Alatalo SL, Suominen H, Cheng S, Janckila AJ, Väänänen HK. Tartrate-resistant acid phosphatase 5b: a novel serum marker of bone resorption. *Journal of Bone and Mineral Research*. 2000;15(7):1337-45.

171. Egbuna OI, Brown EM. Hypercalcaemic and hypocalcaemic conditions due to calcium-sensing receptor mutations. *Best Practice & Research Clinical Rheumatology*. 2008;22(1):129-48.

172. Dhanwal DK. Thyroid disorders and bone mineral metabolism. *Indian Journal of Endocrinology and Metabolism*. 2011;15(Suppl2):S107.

173. Zebaze R, Takao-Kawabata R, Peng Y et al. Increased cortical porosity is associated with daily, not weekly, administration of equivalent doses of teriparatide. *Bone*. 2017;99:80-4.

174. Scott JP, Sale C, Greeves JP, Casey A, Dutton J, Fraser WD. The role of exercise intensity in the bone metabolic response to an acute bout of weight-bearing exercise. *Journal of Applied Physiology*. 2011;110(2):423-32.

175. Sherk VD, Wherry SJ, Barry DW, Shea KL, Wolfe P, Kohrt WM. Calcium Supplementation Attenuates Disruptions in Calcium Homeostasis during Exercise. *Medicine & Science in Sports & Exercise*. 2017.

176. Shea KL, Barry DW, Sherk VD, Hansen KC, Wolfe P, Kohrt WM. Calcium supplementation and PTH response to vigorous walking in postmenopausal women. *Medicine & Science in Sports & Exercise*. 2014;46(10):2007.

177. Warren MP, Gunn JB, Hamilton LH, Warren LF, Hamilton WG. Scoliosis and fractures in young ballet dancers. *New England Journal of Medicine*. 1986;314(21):1348-53.

178. Carbon R, Sambrook P, Deakin V et al. Bone density of élite female athletes with stress fractures. *The Medical Journal of Australia*. 1990;153(7):373-6.

179. Myburgh KH, Hutchins J, Fataar AB, Hough SF, Noakes TD. Low bone density is an etiologic factor for stress fractures in athletes. *Annals of Internal Medicine*. 1990;113(10):754-9.

180. Krishnan V, Bryant HU, MacDougald OA. Regulation of bone mass by Wnt signaling. *The Journal of Clinical Investigation*. 2006;116(5):1202-9.

181. Zagrodna A, Jóźków P, Mędraś M, Majda M, Słowińska-Lisowska M. Sclerostin as a novel marker of bone turnover in athletes. *Biology of Sport*. 2016;33(1):83.

182. Li X, Ominsky MS, Niu QT et al. Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. *Journal of Bone and Mineral Research*. 2008;23(6):860-9.

183. Pickering M-E, Simon M, Sornay-Rendu E et al. Serum Sclerostin Increases After Acute Physical Activity. *Calcified Tissue International*. 2017:1-4.

184. Fazeli PK, Ackerman KE, Pierce L, Guereca G, Bouxsein M, Misra M. Sclerostin and Pref-1 have differential effects on bone mineral density and strength parameters in adolescent athletes compared with non-athletes. *Osteoporosis International*. 2013;24(9):2433-40.

185. Lombardi G, Lanteri P, Colombini A, Mariotti M, Banfi G. Sclerostin concentrations in athletes: role of load and gender. *Journal of Biological Regulators and Homeostatic Agents*. 2012;26(1):157-63.

186. Grasso D, Corsetti R, Lanteri P et al. Bone-muscle unit activity, salivary steroid hormones profile, and physical effort over a 3-week stage race. *Scandinavian Journal of Medicine & Science in Sports*. 2015;25(1):70-80.

187. Milgrom, Finestone A, Novack V et al. The effect of prophylactic treatment with risedronate on stress fracture incidence among infantry recruits. *Bone*. 2004;35(2):418-24.

188. Knapik JJ. The influence of physical fitness training on the manual material handling capability of women. *Applied Ergonomics*. 1997;28(5-6):339-45.

189. Harman E, Frykman P, Palmer C, Lammi E, Reynolds K. Effects of a Specifically Designed Physical Conditioning Program on the Load Carriage and Lifting Performance of Female Soldiers. *DTIC* 1997.

190. Kraemer WJ, Nindl BC, Ratamess NA et al. Changes in muscle hypertrophy in women with periodized resistance training. *Medicine & Science in Sports & Exercise*. 2004;36(4):697-708.

191. Pawlak R, Clasey JL, Palmer T, Symons TB, Abel MG. The effect of a novel tactical training program on physical fitness and occupational performance in firefighters. *Journal of Strength & Conditioning Research*. 2015;29(3):578-88.

192. Nindl BC, Alvar BA, Dudley JR et al. Executive Summary From the National Strength and Conditioning Association's Second Blue Ribbon Panel on Military Physical Readiness: Military Physical Performance Testing. *Journal of Strength & Conditioning Research*. 2015;29:S216-S20.

193. Burgomaster KA, Hughes SC, Heigenhauser GJ, Bradwell SN, Gibala MJ. Six sessions of sprint interval training increases muscle oxidative potential and cycle endurance capacity in humans. *Journal of Applied Physiology*. 2005;98(6):1985-90.

194. Gibala MJ, Little JP, Van Essen M et al. Short-term sprint interval versus traditional endurance training: similar initial adaptations in human skeletal muscle and exercise performance. *The Journal of Physiology*. 2006;575(3):901-11.

195. Knapik JJ, Harman EA, Steelman RA, Graham BS. A systematic review of the effects of physical training on load carriage performance. *Journal of Strength & Conditioning Research*. 2012;26(2):585-97.

196. Hendrickson NR, Sharp MA, Alemany JA et al. Combined resistance and endurance training improves physical capacity and performance on tactical occupational tasks. *European Journal of Applied Physiology*. 2010;109(6):1197-208.

197. Weeks BK, Beck BR. The BPAQ: a bone-specific physical activity assessment instrument. *Osteoporosis International*. 2008;19(11):1567-77.

198. Musgrave KO, Giambalvo L, Leclerc HL, Cook RA, Rosen CJ. Validation of a Quantitative Food Frequency Questionnaire for Rapid Assessment of Dietary Calcium Intake. *Journal of American Dietetics Association*. 1989;89(10):1484-8.

199. Miller T, Association C. *NSCA's Guide to Tests and Assessments*. Human Kinetics; 2012.

200. Seo D-i, Kim E, Fahs CA et al. Reliability of the one-repetition maximum test based on muscle group and gender. *Journal of Sports Science & Medicine*. 2012;11(2):221.

201. Bellace JV, Healy D, Besser MP, Byron T, Hohman L. Validity of the Dexter Evaluation System's Jamar dynamometer attachment for assessment of hand grip strength in a normal population. *Journal of Hand Therapy*. 2000;13(1):46-51.

202. Borg GA. Psychophysical bases of perceived exertion. *Medicine & Science in Sports & Exercise*. 1982;14(5):377-81.

203. Quann EE, Fulgoni VL, Auestad N. Consuming the daily recommended amounts of dairy products would reduce the prevalence of inadequate micronutrient intakes in the United States: diet modeling study based on NHANES 2007–2010. *Nutrition Journal*. 2015;14(1):90.

204. Lewiecki EM, Baim S, Langman CB, Bilezikian JP. The official positions of the International Society for Clinical Densitometry: perceptions and commentary. *Journal of Clinical Densitometry*. 2009;12(3):267-71.

205. Valimaki VV, Alfthan H, Lehmuskallio E et al. Risk factors for clinical stress fractures in male military recruits: a prospective cohort study. *Bone*. 2005; 37(2):267-73.

206. Niculescu D, Dusceac R, Caragheorgheopol A, Ismail G, Poiana C. Parathyroid Hormone-Related Bone Loss in End-Stage Renal Disease: Where to Measure? *Modern Medicine*. 2015;22(4):333-8.

207. Jilka RL. Molecular and cellular mechanisms of the anabolic effect of intermittent PTH. *Bone*. 2007;40(6):1434-46.

208. Galea GL, Lanyon LE, Price JS. Sclerostin's role in bone's adaptive response to mechanical loading. *Bone*. 2017;96:38-44.

209. Kramer I, Halleux C, Keller H et al. Osteocyte Wnt/β-catenin signaling is required for normal bone homeostasis. *Molecular and Cellular Biology*. 2010;30(12):3071-85.

210. Bhatnagar A, Kumar M, Shivanna D, Bahubali A, Manjunath D. High incidence of stress fractures in military cadets during training: A point of concern. *Journal of Clinical and Diagnostic Research.* 2015;9(8):RC01.

211. Dadarya B, Gupta S. Study of Incidence and Pattern of Distribution of Stress Fracture in One Training Center of Paramilitary Force of Central India. *International Journal of Health Sciences and Research*. 2015;5(4):26-30.

212. Beck TJ, Ruff CB, Warden KE, Scott JW, Rao GU. Predicting femoral neck strength from bone mineral data. A structural approach. *Investigative Radiology*. 1990;25(1):6-18.

213. Ahola R, Korpelainen R, Vainionpää A, Leppäluoto J, Jämsä T. Time-course of exercise and its association with 12-month bone changes. *BMC Musculoskeletal Disorders*. 2009;10(1):138.

214. Stone TM, Wingo JE, Young JC, Navalta JW. An Evaluation of Select Physical Activity Exercise Classes on Bone Metabolism. *International Journal of Exercise Science*. 2018;11(2):452.

215. Zribi A, Zouch M, Chaari H et al. Short-term lower-body plyometric training improves whole-body BMC, bone metabolic markers, and physical fitness in early pubertal male basketball players. *Pediatric Exercise Science*. 2014; 26(1):22-32.

216. Davey T, Lanham-New SA, Shaw AM et al. Fundamental differences in axial and appendicular bone density in stress fractured and uninjured Royal Marine recruits—A matched case–control study. *Bone*. 2015;73:120-6.

217. Friedenreich CM, McNeil J. Mapping the historical development of research in physical activity and health: Providing a platform for future research. *Preventative Medicine;* 2018.

218. Medicine ACoS. *ACSM's Guidelines for Exercise Testing and Prescription*. Lippincott Williams & Wilkins; 2013.

219. Reina N, Cavaignac E, Trousdale WH, Laffosse JM, Braga J. Laterality and grip strength influence hand bone micro-architecture in modern humans, an HRpQCT study. *Journal of Anatomy*. 2017;230(6):796-804.

220. Peters MJ, van Nes SI, Vanhoutte EK et al. Revised normative values for grip strength with the Jamar dynamometer. *Journal of the Peripheral Nervous System*. 2011;16(1):47-50.

221. Patterson DD, Peterson DF. Vertical jump and leg power norms for young adults. *Measurement in Physical Education and Exercise Science*. 2004;8(1):33-41.

222. Marshall D, Johnell O, Wedel H. Meta-analysis of how well measures of bone mineral density predict occurrence of osteoporotic fractures. *Bone Mineral Journal*. 1996;312(7041):1254-9.

223. Schott A, Cormier C, Hans D et al. How hip and whole-body bone mineral density predict hip fracture in elderly women: the EPIDOS Prospective Study. *Osteoporosis International*. 1998;8(3):247-54.

224. Hui SL, Slemenda CW, Johnston CC. Baseline measurement of bone mass predicts fracture in white women. *Annals of Internal Medicine*. 1989; 111(5):355-61.

225. Amstrup AK, Sikjaer T, Heickendorff L, Mosekilde L, Rejnmark L. Melatonin improves bone mineral density at the femoral neck in postmenopausal women with osteopenia: a randomized controlled trial. *Journal of Pineal Research*. 2015;59(2):221-9.

226. McClung MR, Grauer A, Boonen S et al. Romosozumab in postmenopausal women with low bone mineral density. *New England Journal of Medicine*. 2014;370(5):412-20.

227. Recker RR, Benson CT, Matsumoto T et al. A randomized, double-blind phase 2 clinical trial of blosozumab, a sclerostin antibody, in postmenopausal women with low bone mineral density. *Journal of Bone and Mineral Research*. 2015; 30(2):216-24.

228. Armamento-Villareal R, Sadler C, Napoli N et al. Weight loss in obese older adults increases serum sclerostin and impairs hip geometry but both are prevented by exercise training. *Journal of Bone and Mineral Research*. 2012; 27(5):1215-21.

229. Hill AV, Lupton H. Muscular exercise, lactic acid, and the supply and utilization of oxygen. *Quarterly Journal of Medicine.* 1923;16:135-71.

230. Liu, Y., Peng, C. H., Wei, S. H., Chi, J. C., Tsai, F. R., & Chen, J. Y. Active leg stiffness and energy stored in the muscles during maximal counter movement jump in the aged. *Journal of Electromyography and Kinesiology*, 2006. *16*(4), 342-351.

231. Steele, James, James Fisher, Jürgen Giessing, and Paulo Gentil. Clarity in reporting terminology and definitions of set endpoints in resistance training. *Muscle & Nerve* 56, no. 3 (2017): 368-374.

232. Amrein, K., Amrein, S., Drexler, C., Dimai, H. P., Dobnig, H., Pfeifer, K., & Fahrleitner-Pammer, A. Sclerostin and its association with physical activity, age, gender, body composition, and bone mineral content in healthy adults. *The Journal of Clinical Endocrinology & Metabolism*, 2012; 97(1), 148-154.

233. Sheng, Z., Tong, D., Ou, Y., Zhang, H., Zhang, Z., Li, S., ... & Liao, E. Serum sclerostin levels were positively correlated with fat mass and bone mineral density in central south Chinese postmenopausal women. *Clinical Endocrinology*, 2012; *76*(6), 797-801.

234. Krause, A., Speacht, T., Govey, P., Zhang, Y., Steiner, J., Lang, C., & Donahue, H. Sarcopenia and Increased Body Fat in Sclerostin Deficient Mice *Journal of Bone Mineral Research* 2012; 29, pp. S8-S9).

Appendix A: IRB Approval Letter

The UNIVERSITY of OKLAHOMA

Institutional Review Board for the Protection of Human Subjects

Initial Submission – Board Approval

Date: November 17, 2017

To: Debra A Bemben, PhD

IRB#: 8600
Meeting Date: 10/30/2017
Approval Date: 11/17/2017
Expiration Date: 09/30/2018

Study Title: A Longitudinal Assessment of Biochemical Markers, Muscular Performance, and Aerobic Capacity in College-Aged ROTC Members

Reference Number: 672638

Study Status: Active - Open

At its regularly scheduled meeting the IRB reviewed the above-referenced research study. Study documents associated with this submission are listed on page 2 of this letter. To review and/or access the submission forms as well as the study documents approved for this submission, open this study from the *My Studies* option, click to open this study, look under Protocol Items to click on the current *Application*, *Informed Consent* and *Other Study Documents*.

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

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

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

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

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

Sincerely,

Martina Jelley, MD, MSPH
Chairperson, Institutional Review Board

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

Appendix B: ROTC Letter of Support

The University of Oklahoma®

DEPARTMENT OF NAVAL SCIENCE

August 1st, 2017

Attn: Dr. Debra Bemben

Department of Health and Exercise Science

University of Oklahoma

1401 Asp Ave.

Norman, OK. 73019

Dear Dr. Bemben,

I am writing to communicate my support for Bree Baker's research on student members of the Naval Reserve Officers Training Corps Unit, University of Oklahoma program. I give her permission to recruit from my unit. The students will participate on a voluntary basis and will not be penalized if they choose to not participate.

If you have any questions, please contact me at (405) 325-1807 or lyle.hall@ou.edu.

Respectfully,

Lyle Hall

Captain Lyle D. Hall, USN

Professor of Naval Science

Department of Naval Science

Naval ROTC Unit, University of Oklahoma

Naval Reserve Officers Training Corps Unit, 290 West Brooks Street, Room 12, Norman, Oklahoma 73019-4102 PHONE: (405) 325-2021 FAX: (405) 325-7639

Appendix C: Informed Consent and HIPAA

701A Consent Version:

IRB Number: 8600

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

A Longitudinal Assessment of Biomarkers, Muscular Performance, and Aerobic Capactity in College Aged ROTC Members

Principal Investigator: Debra Bemben, PhD

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 trial/study because you are either 1) a member of a collegiate ROTC program or 2) a recreationally active participant.

Why Is This Study Being Done?
The purpose of this study is to track how eight weeks of military training affects ROTC members biochemical markers, body composition, and physical performance as compared to physically active non-ROTC members. This study will potentially allow researchers to identify novel biomarkers that can be used as additional tools for bone injury assessment in military personnel. Additionally, this study will allow for the examination of the relationships between these biomarkers and measures of body composition and physical performance.

How Many People Will Take Part In The Study?
Approximately 25 ROTC members and 25 control participants will take part in this study.

What Is Involved In The Study?
If you take part in this study, seven visits will be needed. The first visit consisting of the following tests in order to determine history relating to bone health and familiarize you to the physical performance tests. This visit will last about 1 hour.

- Informed consent – must sign and date an informed consent form (this document) stating that you understand all procedures and your rights as a participant.
- Health Status Questionnaire – you may be excluded from the study if any answer on this questionnaire indicates you may not be eligible for this study.
- Bone Specific Physical Activity Questionnaire – this questionnaire will be used to determine if 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, healthy bone.
- Menstrual History Questionnaire- will determine any menstrual abnormalities that might impact bone health in female participants.
- Physical Activity Readiness Questionnaire- will determine if you are healthy enough to engage in exercise.

- Auxiliary Training Questionnaire- will determine other modes of exercise you regularly engage in.
- You will become accustomed to the methods of the jump platform, grip strength test, bench press, decline leg press, and treadmill graded exercise test.

The second and fifth visits will consist of the following tests to evaluate your biochemical markers, body composition, and muscular strength and power. These visits will take about 2.5 hours.

- A blood draw (approximately 2 teaspoons per sample) will be performed by a nurse or phlebotomist. The venipuncture site will be covered with a cotton ball and the arm wrapped. All samples are obtained in the morning, after an 8-hour overnight fast and abstaining from exercise the previous 24 hours.
- Urine Test – will be completed in order to determine that females are not pregnant and determine if your hydration status is within normal ranges.
- Height and Weight – your height and weight will be measured.
- Series of Dual Energy X-ray Absorptiometry (DXA) scans – will be used to determine the bone mineral density of the total body, lumbar spine, the right and left hips. These tests are non-invasive and will take approximately 35 minutes to complete. DXA is a radiation procedure and is for research purposes only. There are risks associated with DXA which will be addressed below.
- Series of peripheral Quantitative Computed Tomography (pQCT) scans – These scans will include 6 scans on both lower legs. 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. The pQCT utilizes radiation and is for research purposes alone. There are risks associated with pQCT which will be addressed below.
- Grip Strength Testing- will be completed to record maximum grip strength.
- Jump Testing- will be used to measure jump height, air time, power and velocity.
- Decline Leg Press- This is a lower body exercise that requires you to push as much weight away from your body as possible with your legs.
- Bench Press- This is an upper body exercise that requires you to push as much weight away from your chest as possible with your arms.

The fourth visit will repeat the same urine, height and weight measurements, in addition to a total body DXA scan, and the same grip, jump, bench, and leg press measures as previously described. This visit will last approximately 60 minutes.

The third and sixth visits will be an aerobic capacity test, lasting 30 minutes.

- Aerobic Capacity-You will complete a graded exercise test where you begin by walking on the treadmill, as time continues you will begin to move faster and at a greater incline for as long as you can tolerate. You will be wearing a heart rate monitor and facemask so researchers can collect your expired breaths.

How Long Will I Be In The Study?
The study will span 8 weeks and require approximately 8 hours total. The first visit will take approximately 1 hour and consist of the consenting process, questionnaires and a familiarization with the testing methods. The second and fifth visits will take approximately 2.5 hours each and will consist of the blood draw, DXA and pQCT bone scans, grip strength, jump test, bench press, leg press. Visit four is an abbreviated version lasting approximately 1 hour. The third and sixth visits are about 30 minutes each, consisting of an aerobic capacity test.

There may be anticipated 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
- presence of metal implants
- recent injuries
- Physical Activity Status

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

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

- Risks and side effects related to having a pQCT and DXA scan include radiation exposure from 9 DXA scans and 12 pQCT scans, which are types of x-ray procedures. These procedures are for research only and not needed for your medical care. The amount of additional radiation to which you will be exposed is approximately 1% of the amount of radiation to which we are exposed annually from background sources such as the Earth and Sun. In addition to any radiographic procedures that are being done as part of this research, you may also be exposed to radiation from procedures that are part of your normal care. The risk from radiation exposure increases over your lifetime as you receive additional exposure to radiation.
- Risks and side effects related to blood draws include discomfort at the site of the needle stick and possible bruising during and after the blood draw. Only qualified personnel will conduct all blood sampling methods.
- Risks and side effects related to physical performance testing include acute and delayed muscle soreness, musculoskeletal injury, discomfort during exercise, feeling tired, lightheaded, or faint. Researchers will make sure that you have eaten food and are hydrated prior to exercise to help minimize these symptoms.
- If you are a female, you should not become pregnant while in this study. Participating in the DXA and pQCT scans involved in this study while pregnant may involve risks to an embryo or fetus, including birth defects. In order to reduce your risk of pregnancy, you or your partner should use some of the acceptable methods of birth control listed below.

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

- o An approved oral contraceptive (birth control pill) or Intra-uterine device (IUD)
- o Hormone implants or contraceptive injection (Depo-Provera)
- o Barrier methods (diaphragm with spermicidal gel or condoms)
- o Transdermal patches or vaginal rings (birth control ring) or sterilization

If you become pregnant or suspect that you are pregnant during this study, you should immediately inform the study personnel in order to complete an additional test. If pregnancy is confirmed, you may be withdrawn from the study.

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

What Other Options Are There?
Your alternative is to not participate in this study.

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 outside the OUHSC that may inspect and/or copy your research records for quality assurance and data analysis. These organizations include the US Food & Drug Administration and other regulatory agencies and the Department of Health & Exercise Science at the University of Oklahoma-Norman campus. The OUHSC Human Research Participant Program office, the OUHSC Institutional Review Board, and the OUHSC Office of Compliance may also inspect and/or copy your research records for these purposes.

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

Will I Be Paid For Participating in This Study?
Participants will be provided a t-shirt as compensation for their time.

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 405-325-5211 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)	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: **A Longitudinal Assessment of Biochemical Markers, Muscular Performance, and Aerobic Capacity in College Aged ROTC Members**

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

Address: **1401 Asp Avenue, Norman, OK, 73071**

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

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

PHI To Be Used or Shared. Federal law requires that researchers get your permission (authorization) to use or share your PHI. If you give permission, the researchers may use or share with the people identified in this Authorization any PHI related to this research from your medical records and from any test results. Information used or shared may include all information relating to any tests, procedures, surveys, or interviews as outlined in the consent form; medical records and charts; name, address, telephone number, date of birth, race, government-issued identification numbers, and nothing else.

Purposes for Using or Sharing PHI. If you give permission, the researchers may use your PHI to investigate the changes in biochemical serum blood markers, body composition, and muscular performance and aerobic capacity in college aged ROTC members in response to an eight week military training period as compared to a matched control population.

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.

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

[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.**

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.

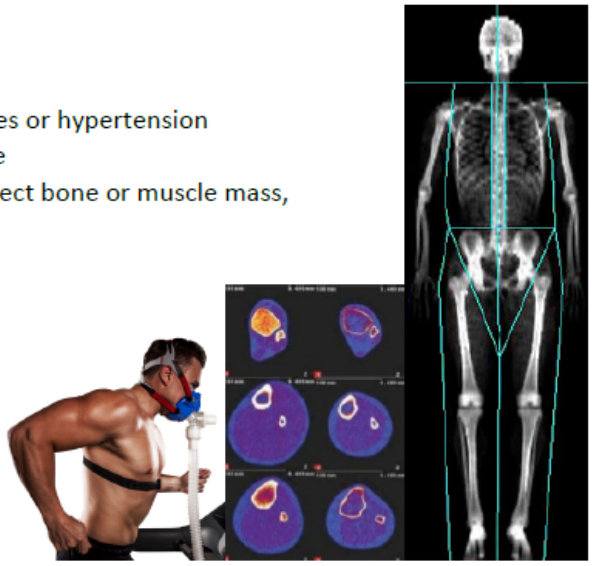
Appendix D: Recruitment Flier

Want Body Composition and Physical Performance Testing?

A Longitudinal Assessment of Biochemical Markers, Muscular Performance, and Aerobic Capacity in College-Aged ROTC Members

To Participate

- Males and females aged 18-30 years
- Be physically active at least 3x/week
- Non smokers who are free of diabetes or hypertension
- No metal implants in the hip or spine
- Not taking medications known to affect bone or muscle mass, such as corticosteroids



Required Testing

- 6 visits over 8 weeks
- About 8 hours total time
- Blood draws at OU Goddard
- DXA & pQCT scans
- Power and Strength testing
- Aerobic capacity testing

Possible risks are associated with radiation exposure, blood draw and exercise.

Tests will take place at Huston Huffman Center Bone Density Lab,
University of Oklahoma Norman Campus

If you are eligible and interested, please contact:
Bree Baker at 719-429-2690, Bree.Baker@OU.edu
Department of Health and Exercise Science

The University of Oklahoma is an equal opportunity institution. IRB 8600

Name: Bree Baker
Phone: 719-429-2690
Email: bree.baker@ou.edu

Name: Bree Baker
Phone: 719-429-2690
Email: bree.baker@ou.edu

Appendix E: Screening Material and Questionnaires

Screening Checklist –ROTC

A Longitudinal Assessment of Biochemical Markers, Muscular Performance, and Aerobic Capacity in College Aged ROTC Members

Name:_____ Date:_____

Does the subject meet the inclusion criteria for the study?

	YES	NO
Age between 18 and 30 years		
Actively participating in a collegiate ROTC program.		
No current injury or illness preventing strenuous physical activity.		

Does the subject have any exclusion criteria?

	YES	NO
Women who are pregnant or planning to become pregnant.		
Body mass over 300 pounds.		
Disease known to affect BMD: -Parathyroid disease - Diabetes -Bone cancer or metabolic disease -Bladder or kidney stones		
Medications known to affect bone mineral density such as: -Corticosteroids (asthma) -Anabolic steroids		
Joint replacement or metal implants in the legs, hips, or spine.		
Regularly consumed any form of tobacco in the past 5 years.		

STOP HERE

Is the subject qualified for the study (circle one)? YES NO

Primary Investigator approval
Dr. Debra Bemben

Signature: _____ Date: _____

IRB NUMBER: 8600
IRB APPROVAL DATE: 11/17/2017

Screening Checklist –CONTROL

A Longitudinal Assessment of Biochemical Markers, Muscular Performance, and Aerobic Capacity in College Aged ROTC Members

Name:_____ Date:_____

Age (years):_____Body Weight (pounds):_____

Does the subject meet the inclusion criteria for the study?

	YES	NO
Age between 18 and 30 years		
Physically active at least 3x/week		
No current injury or illness preventing strenuous physical activity		
Matches sex, ± 2yrs, and ±5lbs of a ROTC member		

Does the subject have any exclusion criteria?

	YES	NO
Participating in a ROTC program.		
Women who are pregnant or planning to become pregnant.		
Body mass over 300 pounds.		
Disease known to affect BMD: -Parathyroid disease - Diabetes -Bone cancer or metabolic disease -Bladder or kidney stones		
Medications known to affect bone mineral density such as: -Corticosteroids (asthma) -Anabolic steroids		
Joint replacement or metal implants in the legs, hips, or spine.		
Regularly consumed any form of tobacco in the past 5 years.		

STOP HERE

Is the subject qualified for the study (circle one)? YES NO

Primary Investigator approval
Dr. Debra Bemben

Signature: _____ Date: _____

IRB NUMBER: 8600
IRB APPROVAL DATE: 11/17/2017

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 Ethnicity

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

9. Circle operations you have had:
Back Heart (MC) Kidney Eyes Joint Neck

Ears Hernia Lung Other _____ **NONE**

IRB NUMBER: 8600
IRB APPROVAL DATE: 11/17/2017

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	Diuretic (MC)
Depression	Heart-rhythm medication (MC)	Other _____
Diabetic pill	Insulin (MC)	**NONE**
Digitalis (MC)	Nitroglycerin (MC)	

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

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

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

Part 3. Health-related behavior

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

14. If you are a smoker, indicate number smoked per day:
Cigarettes: 40 or more 20-39 10-19 1-9
Cigars or pipes only: 5 or more or any inhaled Less than 5, none inhaled

15. Weight now: _____lb. One year ago: _____lb. Age 21 (if applicable): _____lb.

16. Do you regularly engage in strenuous exercise or hard physical labor? YES NO

17. Do you exercise or labor at least three times a week? YES NO

Physical Activity Readiness
Questionnaire - PAR-Q
(revised 2002)

PAR-Q & YOU

(A Questionnaire for People Aged 15 to 69)

Regular physical activity is fun and healthy, and increasingly more people are starting to become more active every day. Being more active is very safe for most people. However, some people should check with their doctor before they start becoming much more physically active.

If you are planning to become much more physically active than you are now, start by answering the seven questions in the box below. If you are between the ages of 15 and 69, the PAR-Q will tell you if you should check with your doctor before you start. If you are over 69 years of age, and you are not used to being very active, check with your doctor.

Common sense is your best guide when you answer these questions. Please read the questions carefully and answer each one honestly: check YES or NO.

YES	NO	
☐	☐	**1. Has your doctor ever said that you have a heart condition and that you should only do physical activity recommended by a doctor?**
☐	☐	**2. Do you feel pain in your chest when you do physical activity?**
☐	☐	**3. In the past month, have you had chest pain when you were not doing physical activity?**
☐	☐	**4. Do you lose your balance because of dizziness or do you ever lose consciousness?**
☐	☐	**5. Do you have a bone or joint problem (for example, back, knee or hip) that could be made worse by a change in your physical activity?**
☐	☐	**6. Is your doctor currently prescribing drugs (for example, water pills) for your blood pressure or heart condition?**
☐	☐	**7. Do you know of any other reason why you should not do physical activity?**

If you answered

YES to one or more questions

Talk with your doctor by phone or in person BEFORE you start becoming much more physically active or BEFORE you have a fitness appraisal. Tell your doctor about the PAR-Q and which questions you answered YES.

- You may be able to do any activity you want — as long as you start slowly and build up gradually. Or, you may need to restrict your activities to those which are safe for you. Talk with your doctor about the kinds of activities you wish to participate in and follow his/her advice.
- Find out which community programs are safe and helpful for you.

NO to all questions

If you answered NO honestly to all PAR-Q questions, you can be reasonably sure that you can:

- start becoming much more physically active – begin slowly and build up gradually. This is the safest and easiest way to go.
- take part in a fitness appraisal – this is an excellent way to determine your basic fitness so that you can plan the best way for you to live actively. It is also highly recommended that you have your blood pressure evaluated. If your reading is over 144/94, talk with your doctor before you start becoming much more physically active.

DELAY BECOMING MUCH MORE ACTIVE:

- if you are not feeling well because of a temporary illness such as a cold or a fever – wait until you feel better; or
- if you are or may be pregnant – talk to your doctor before you start becoming more active.

PLEASE NOTE: If your health changes so that you then answer YES to any of the above questions, tell your fitness or health professional. Ask whether you should change your physical activity plan.

Informed Use of the PAR-Q: The Canadian Society for Exercise Physiology, Health Canada, and their agents assume no liability for persons who undertake physical activity, and if in doubt after completing this questionnaire, consult your doctor prior to physical activity.

No changes permitted. You are encouraged to photocopy the PAR-Q but only if you use the entire form.

NOTE: If the PAR-Q is being given to a person before he or she participates in a physical activity program or a fitness appraisal, this section may be used for legal or administrative purposes.

"I have read, understood and completed this questionnaire. Any questions I had were answered to my full satisfaction."

NAME _____

SIGNATURE _____ DATE _____

SIGNATURE OF PARENT or GUARDIAN (for participants under the age of majority) _____ WITNESS _____

Note: This physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if your condition changes so that you would answer YES to any of the seven questions.

Supported by: Health Canada Santé Canada

continued on other side...

Auxiliary Training Questionnaire

Participant ID: _____ Date_____

ROTC members please only include exercise outside of ROTC training regimen

On average how many days per week are you physically active?_____

About how long have you been this physically active?_____

Do you engage in **resistance training**? _____Yes_____No

Type of exercise _____

Frequency per week_____ Duration per session _____Intensity (Sets/Reps)_____

Type of exercise _____

Frequency per week_____ Duration per session _____Intensity (Sets/Reps)_____

Do you engage in **cardiovascular training**? _____Yes_____No

Type of exercise _____

Frequency per week_____ Duration per session _____Intensity _____

Type of exercise _____

Frequency per week_____ Duration per session _____Intensity _____

Do you engage in **group fitness classes**? _____Yes_____No

Type of exercise _____

Frequency per week_____ Duration per session _____Intensity _____

Please use the space below to describe any other physical activity you regularly engage in.

Have you ever had a musculoskeletal injury such as shin splints, stress fractures, or muscle strains associated with your physical activity regimen? If so please describe the injury.

IRB NUMBER: 8600
IRB APPROVAL DATE: 11/17/2017

Bone-Specific Physical Activity Questionnaire (BPAQ)
OU Bone Density Research Laboratory

Participant ID: _____ Date: _____

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

Sport/Activity	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	26	27	28	29	30

2. Please list the sports or other physical activities (be as specific as possible) you participated in regularly in the past 12 months and indicate the average frequency (sessions per week)? On the back of this page is a list of activities you may use as a reference.

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

IRB NUMBER: 8600
IRB APPROVAL DATE: 11/17/2017

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

CALCIUM INTAKE ESTIMATION

Participant ID:_____ 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	Score	Food Type	serving size	servings/week	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, etc.	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	Score	Food Type	serving size	servings/week	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 Density Research Laboratory
Department of Health and Exercise Science
University of Oklahoma

MENSTRUAL HISTORY QUESTIONNAIRE

Participant ID:_____Date:_____

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

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.

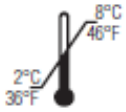
Appendix F: Assay Kit Instructions

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.



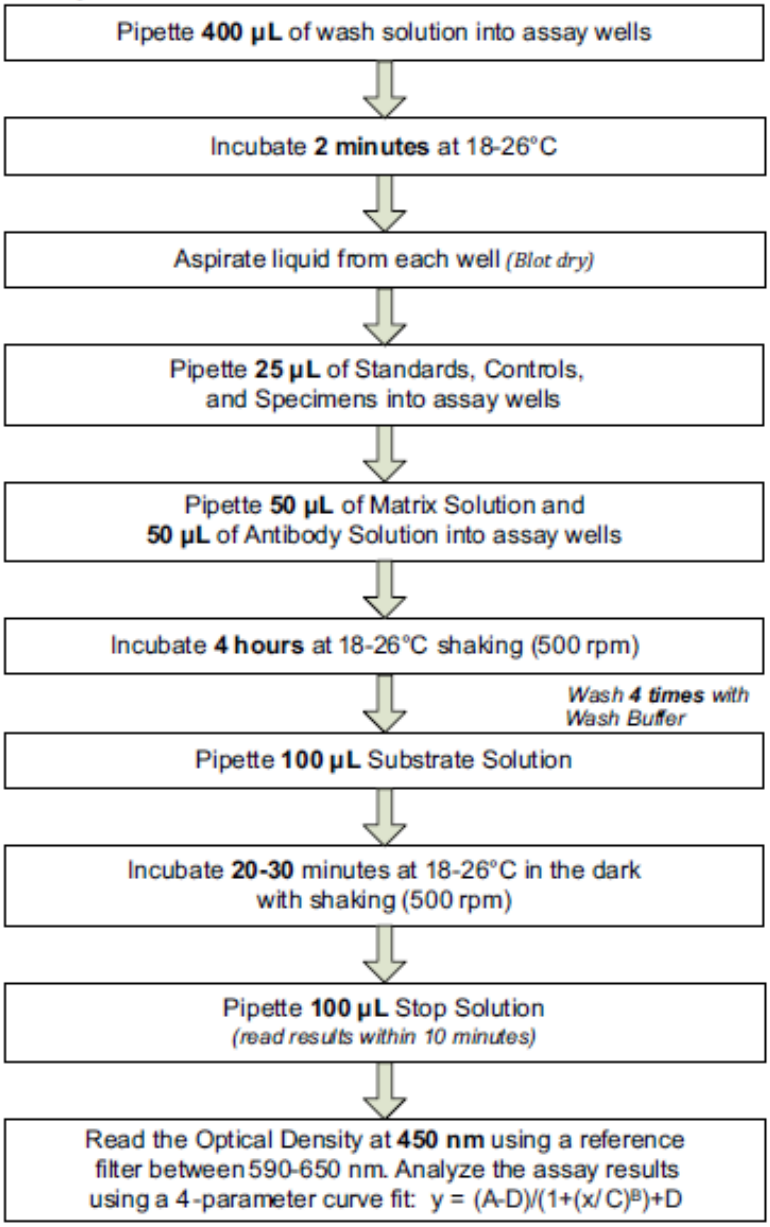
REF TE1023HS

TECOmedical Sclerostin HS EIA Summary

Reagent, Standards, Controls, and Sample Preparation

- ❑ Dilute Wash Buffer Concentrate **1:20** with DI Water.

Assay Procedure



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 periprosthetic 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.

In Step 2, a chromogenic enzyme substrate is added to each microassay well. The bound HRP-conjugate reacts with the substrate, forming a blue color. After incubation the enzyme reaction is stopped chemically, the color changes to yellow, and the color intensity is measured spectrophotometrically at 450 nm with a 590-650 nm reference filter. The color intensity of the reaction mixture is proportional to the concentration of Sclerostin present in the test specimens, Standards, and Controls.

REAGENTS AND MATERIALS PROVIDED

96 Assays for Human Sclerostin

Human Sclerostin HS Enzyme Immunoassay kit contains the following:

A | F Sclerostin Standards — **Parts 5178 – 5183** — **0.5 mL each**
Concentration: 0, 0.05, 0.2, 0.5, 1.5, 3 ng/mL (0, 2.2, 8.8, 22, 66, 132 pmol/L)
Ready to use. Each contains recombinant protein with assigned Sclerostin concentration (ng/mL) based on amino acid analysis, protein stabilizers, 0.06% BND, 0.05% Tween-20®

L Sclerostin Low Control — **Part 5184** — **0.5 mL**
Ready to use. Contains recombinant protein with assigned Sclerostin concentration (ng/mL), protein stabilizers, 0.06% BND, 0.05% Tween-20®

H Sclerostin High Control — **Part 5185** — **0.5 mL**
Ready to use. Contains recombinant protein with assigned Sclerostin concentration (ng/mL), protein stabilizers, 0.06% BND, 0.05% Tween-20®

❶ Microassay Plate — **Part 4634** — **12 x 8 wells**
Eight-well strips coated with Streptavidin in a resealable foil pouch

❷ Stop Solution — **Part A9947** — **12 mL**
Contains 1M (4%) Hydrochloric Acid

❸ 20X Wash Buffer Concentrate — **Part A9957** — **50 mL**
Contains phosphate buffered saline (PBS), 1.0% Tween-20® and 0.035% Proclin® 300

❹ Sample Diluent — **Part 5186** — **5 mL**
Contains protein stabilizers, 0.06% BND, 0.05% Tween-20®

❺ Matrix Solution — **Part 5188** — **7 mL**
Contains protein stabilizers, 0.12% BND

❻ Sclerostin Antibody Solution — **Part 5191** — **7 mL**
Ready to use. Contains biotin-conjugated polyclonal anti-human Sclerostin antibody and horseradish peroxidase-conjugated monoclonal anti-human Sclerostin antibody, protein stabilizers, 0.06% BND, 0.05% Tween-20®

❼ TMB Substrate — **Part 5190** — **12 mL**
Ready to use. Contains 3,3',5,5'-tetramethylbenzidene (TMB) and Hydrogen Peroxide (H_2O_2)

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 Biomedical Laboratories."[22-23]
3. Material 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.

12. Proper collection and storage of test specimens are essential for accurate results (see *Specimen Handling And Preparation*).
13. Avoid microbial or cross-contamination of specimens or reagents.
14. Test each sample in duplicate.
15. Do not use any single microassay well for more than one test.
16. Using incubation times and temperatures other than those indicated in the Procedure section may give erroneous results.
17. The TMB Substrate must be protected from light and contact with metal or rubber during storage and incubation. Avoid contact with eyes, skin, and clothing. If contact is made, immediately rinse affected area with water.
18. Do not allow microassay wells to dry once the assay has begun.
19. When removing liquid from the microassay wells, do not scrape or touch the bottom of the wells.
20. Hyperlipemic or contaminated specimens may give erroneous results.
21. To avoid aerosol formation during washing, use an apparatus to aspirate the wash fluid into a bottle containing household bleach.
22. A wash bottle or automated filling device should be used to wash the plate (*Assay Procedure, step 7*). For best results, do not use a multichannel pipette to wash the microassay plate.
23. Dispose of containers and unused contents in accordance with Federal, State, and Local regulations.
24. For more information, consult Safety Data Sheet available on quidel.com.

STORAGE

Store the unopened kit and unused kit components at 2-8°C.

INDICATIONS OF INSTABILITY OR DETERIORATION OF REAGENTS

Cloudiness or discoloration of the diluted Wash Buffer indicates a deterioration of this reagent. If either of these conditions occur, the solution should be discarded.

REAGENT PREPARATION

Bring all reagents and materials to 18–26°C before use.

After removing the needed reagents and materials, return the unused items to their appropriate storage temperatures (see *Storage*).

Microassay Strips

Determine the number of strips needed for the assay. Assay the Standards, Controls and Samples as quickly as possible (< 15 minutes) and, respectively, in the same order in duplicate. Remove the unneeded strips, place them in the storage bag, reseal the bag, and return it to 2-8°C. Secure the strips to be used in the assay in the assay plate frame.

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.

2. Prepare the microassay strips as follows:
   a. Using a wash bottle or automated plate washing device, add approximately 400 µL Wash Buffer to each well.
   b. Incubate the wells for two minutes at 18-26°C.
   c. Aspirate the contents from each well.
   d. Invert the plate and tap firmly on absorbent paper to remove any remaining liquid.
3. Add 25 µL Standards, Controls, or specimens to the assigned duplicate wells.
4. Add 50 µL Matrix Solution to each well. Use of a multichannel pipette is recommended.
5. Add 50 µL Antibody Solution to each well. The entire plate must be loaded within 15 minutes of loading the first sample onto the plate. Use of a multichannel pipette is recommended.
6. Cover the wells with sealing tape, and incubate for 4 hours at 18-26°C with shaking (500 rpm).
7. Wash the microassay wells a total of 4 times using the following procedure:
   a. Aspirate the contents from each well.
   b. Using a wash bottle or automated plate washing device, add approximately 400 µL diluted Wash Buffer to each well. **NOTE:** Use of an automatic plate washer is recommended. The washer should be primed with Wash Buffer immediately before beginning wash procedure. **DO NOT** use a multichannel pipette for washing.
   c. Immediately aspirate the contents from each well.
   d. Invert the plate, and tap firmly on absorbent paper to remove any remaining liquid.
   e. **Repeat steps b-d three additional times for a total of four washes.**
   f. After the fourth wash cycle, invert the plate and tap firmly on absorbent paper to remove any remaining liquid.
8. Immediately following the wash procedure, dispense 100 µL of the TMB Substrate Solution into each well. Use of a multichannel pipette is recommended.
9. Incubate the microassay strips at 18-26°C in the dark for 20-30 minutes with shaking (500 rpm).
10. Add 100 µL of Stop Solution to each well to stop the enzymatic reaction. The Stop Solution should be added to the wells in the same order and at the same rate that the Substrate Solution had been added. Use of a multichannel pipette is recommended.
11. Gently tap the plate on the bench top to disperse the color development completely and evenly.

    NOTE: Optimal results may be obtained by using the plate reader's auto–mix function (if available) just prior to reading the plate.
12. Determine the absorbance reading at 450 nm (using a reference filter between 590-650 nm) for each test well within 10 minutes after the addition of the Stop Solution (step 11).

13. Determine the concentration of Samples and Controls from the standard curve.
14. Dispose of the remaining specimens and controls and the used microassay strips (see *Warnings And Precautions*).

QUALITY CONTROL

The Certificate of Analysis included in this kit is lot specific and is to be used to verify that the results obtained by your laboratory are similar to those obtained at Quidel Corporation. The optical density values provided are intended as a guideline only. The results obtained by your laboratory may differ.

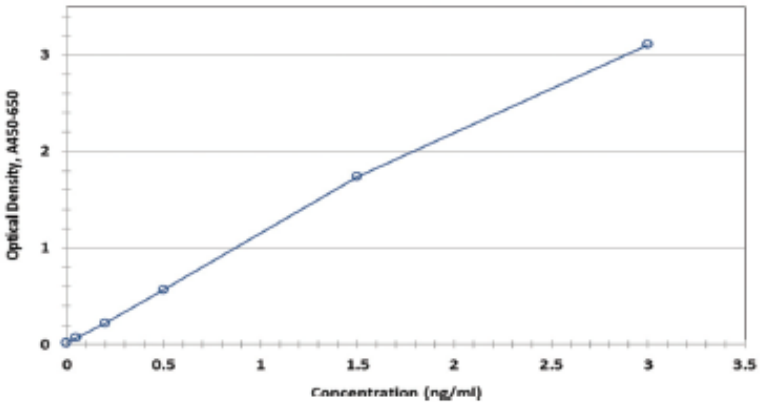
Quality control ranges are provided. The control values are intended to verify the validity of the curve and sample results. Each laboratory should establish its own parameters for acceptable assay limits. If the control values are NOT within your laboratory's acceptance limits, the assay results should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

INTERPRETATION OF RESULTS

Use of the Standard Curve

The standard curve for the Sclerostin HS EIA is generated using the $A_{450-650}$ values for each Standard (on the y axis) and the assigned concentration for each Sclerostin Standard (on the x axis). After 4-parameter regression, the generated standard curve must meet the validation requirements (see below). Most plate-reading software and computers are capable of performing these calculations.

Figure 1: Representative Standard Curve



Sample	$A_{450-650}$	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.

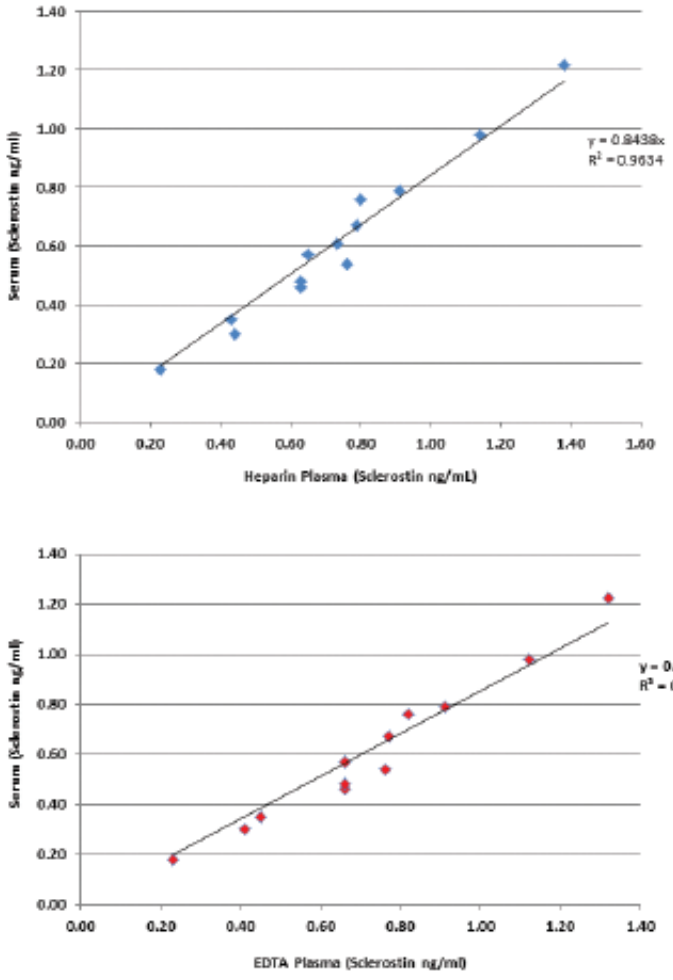
OBSERVED VALUES

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	0.45	0.15
Postmenopausal women	20	0.51	0.14
Men	11	0.59	0.13

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



Sample	n	Mean (ng/mL)	SD (ng/mL)
Serum	9	1.15	0.78
Heparin plasma	9	1.34	0.88
EDTA plasma	9	1.37	0.90

NOTE: 20% higher Sclerostin values have been observed in Heparin and EDTA plasma, compared with serum.

PERFORMANCE OF THE TEST

Limits (as determined by Point-to-Point for LOD and LLOQ)

LOD: The limit of detection (LOD) for the Sclerostin HS EIA is 0.009 ng/mL, determined by the upper 3SD limit in a zero standard study.

LLOQ: The lower limit of quantitation (LLOQ) for the Sclerostin HS EIA is 0.058 ng/mL, the lowest concentration on the standard curve that met CLSI criteria for accuracy and precision.

ULOQ: The upper limit of quantitation (ULOQ) for the Sclerostin HS EIA is 3.5 ng/mL, the highest concentration that met CLSI criteria for accuracy and precision.

Interfering Substances

The following substances were tested in the Sclerostin HS EIA and found to not interfere with the assay using serum samples:

Substance	Concentration
Bilirubin	40 mg/dL
Hemoglobin	500 mg/dL
Triglycerides	3000 mg/dL
Glucose	1200 mg/dL
Cholesterol	500 mg/dL
Albumin	6000 mg/dL
Gamma Globulin	6000 mg/dL

Precision

Intra- and inter-assay precision was determined by assaying 20 replicates of 4 serum sample in 10 different assays.

Sample	Sclerostin (ng/mL)	Within-run[1] C.V. (%)	Between-run[2] C.V. (%)
Serum 1	0.67	4.0	4.8
Serum 2	1.80	4.2	4.4
Serum 3	2.44	3.9	4.3
Serum 4	1.12	3.7	4.5

[1]n = 20 replicates [2]n = 10 runs

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)[3]	Expected Sclerostin (ng/mL)[3]	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

[3]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

No cross-reaction with other species.

ASSISTANCE

To place an order or for technical assistance, please contact a Quidel Representative at 800.874.1517, Monday through Friday, between 8:00 a.m. and 5:00 p.m., Eastern Time. Orders may also be placed by e-mail at custserv@quidel.com or by fax at 740.592.9820. For services outside the U.S., please contact your local distributor.

Additional information about Quidel and Quidel's products and distributors can be found on our website at quidel.com.

REFERENCES

1. Ott S.M. 2005. "Sclerostin and Wnt signaling-the pathway to bone strength." *J Clin Endocrinol Metab*. 90(12):6392-6395.
2. Semenov M, Tamai K, He X. 2005. "SOST is a ligand for LRP5/LRP6 and a Wnt signaling inhibitor." *J Biol Chem*. 280(29):26770-26775.
3. Li X, Zhang Y, Kang H, Liu W, Liu P, Zhang J, Harris S.E., Wu D. 2005. "Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling." *J Biol Chem*. 280(20):19883-19887.
4. ten Dijke P, Kraus C, de Gorter D.J., Lowik C.W., van Bezooijen R.L. 2008. "Osteocyte-derived sclerostin inhibits bone formation: its role in bone morphogenic protein and Wnt signaling." *J Bone Joint Surg Am*. 90(Suppl. 1):31-35.
5. Winkler D.G., Sutherland M.K., Geoghegan J.C., Yu C., Hayes T., Skonier J.E., Shpekor D., Jonas M., Kovacevich B.R., Staehling-Hampton K., Appleby M., Brunkow M.E., Latham J.A. 2003. "Osteocyte control of bone formation via sclerostin, a novel BMP antagonist." *The Embo J*. 22(23):6267-6276.
6. Krause C., Korchynskyi O., de Rooij K., Weidauer S.E., de Gorter D.J., van Bezooijen R.L., Hatsell S., Economides A.N., Mueller T.D., Lowik C.W., ten Dijke P. 2010. "Distinct modes of inhibition by sclerostin on bone morphogenic protein and Wnt signaling pathways." *J Biol Chem*. 285(53):41614-41626.
7. Sutherland M.K., Geoghegan J.C., Yu C., Winkler D.G., Latham J.A. 2004. "Unique regulation of SOST, the sclerosteosis gene, by BMPs and steroid hormones in human osteoblasts." *Bone* 35(2):448-454.
8. Keller H., Kneissel M. 2005. "SOST is a target gene for PTH in bone." *Bone* 37(2):148-158.
9. Bellido T., Ali A.A., Gubrij I., Plotkin L.I., Fu Q., O'Brien C.A., Manolagas S.C., Jilka R.L. 2005. "Chronic elevation of paraghyroid hormone in mice reduces expression of sclerostin by osteocytes: novel mechanism for hormonal control of osteoblastogenesis." *Endocrinology* 146(11):4577-4583.
10. Silvestrini G., Ballanti P., Leopizzi M., Sebastiani M., Berni S., Di Vito M., Bonucci E. 2007. "Effects of intermittent parathyroid hormone (PTH) administration on SOST mRNA and protein in rat bone." *J Molec Hist*. 38(4)l261-269.

11. 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

12. 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.

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

14. 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.

15. 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.

16. 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.

17. 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.

18. 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.

19. 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.

20. 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.

21. Ettenberg S.A., Charlat O., Daley M.P., Liu S., Vincent K.J., Stuart D.D., Schuller A.G., Yuan J., Ospina B., Green J., Yu Q., Walsh R., Li S., Schmitz R., Heine H., Bilic S., Ostrom L., Mosher R., Hartlepp K.F., Zhu Z., Fawell S., Yao Y-M., Stover D., Finan P.M., Porter J.A., Sellers W.R., Klagge I.M., Cong F. 2010. "Inhibition of tumorigenesis driven by different Wnt proteins requires blockade of ligand-binding regions by LRP6 antibodies." *Proc Natl Acad Sci.* 107(35):15473-15478.

22. U.S. Department of Health and Human Services Centers for Disease Control and Prevention and National Institutes of Health. 2007. *Biosafety In Microbiological And Biomedical Laboratories (BMBL) 5TH Edition.* Washington: U.S. Government Printing Office. http://www.cdc.gov/od/ohs/biosfty/bmbl5/bmbl5toc.htm.

23. Centers for Disease Control. 1987. "Recommendations for prevention of HIV transmission in health-care settings." *MMWR.* 36 (suppl. No. 2S):001.

Catalog Number

Manufacturer

Temperature Limitation

Biological risks

Research Use Only

Consult Instructions for Use

Contains sufficient for <n> tests

Contents / contains

REF **TE1023HS – TECOmedical Sclerostin HS Enzyme Immunoassay Kit**

Manufactured by

Quidel Corporation | 2005 E. State St. Suite 100
Athens, OH 45701 USA | quidel.com/spg

For

TECOmedical Group

PIT102300EN01 (08/16)

CE

Revised 3 Dec. 2010 rm (Vers. 6.1) IVD

Please use only the valid version of the package insert provided with the kit.

1 NAME AND INTENDED USE

The DRG Intact-PTH ELISA is intended for the quantitative determination of Intact-PTH (Parathyroid Hormone) in human serum.
This assay is intended for *in vitro* diagnostic use.

2 SUMMARY AND EXPLANATION

PTH (Parathyroid hormone, Parathormone, Parathyrin) is biosynthesized in the parathyroid gland as a pre-proparathyroid hormone, a larger molecular precursor consisting of 115 amino acids. Following sequential intracellular cleavage of a 25-amino acid sequence, preproparathyroid hormone is converted to an intermediate, a 90-amino acid polypetide, proparathyroid hormone. With additional proteolytic modification, proparathyroid hormone is then converted to parathyroid hormone, an 84 amino acid polypeptide. In healthy individuals, regulation of parathyroid hormone secretion normally occurs via a negative feedback action of serum calcium on the parathyroid glands. Intact PTH is biologically active and clears very rapidly from the circulation with a half-life of less than four minutes[1]. PTH undergoes proteolysis in the parathyroid glands, but mostly peripherally, particularly in the liver but also in the kidneys and bone, to give N-terminal fragments and longer lived C-terminal and midregion fragments. In subjects with renal insufficiency, C-terminal and midregion PTH assays typically give elevated PTH results, as reflected by impaired renal clearance[2].

3 CLINICAL SIGNIFICANCE

Intact PTH assays are important for the differentiation of primary hyperparathyroidism from other (non-parathyroid-mediated) forms of hypercalcemia, such as malignancy, sarcoidosis and thyrotoxicosis[2]. The measurement of parathyroid hormone is the most specific way of making the diagnosis of primary hyperparathyroidism. In the presence of hypercalcemia, an elevated level of parathyroid hormone virtually establishes the diagnosis. In over 90% of patients with primary hyperparathyroidism, the parathyroid hormone will be elevated[3].
The most common other cause of hypercalcemia, namely hypercalcemia of malignancy, is associated with suppressed levels of parathyroid hormone[3] or PTH levels within the normal range[4]. When intact PTH level is plotted against serum calcium, the intact PTH concentration for patients with hypercalcemia of malignancy is almost always found to be inappropriately low when interpreted in view of the elevated serum calcium[3,4,5].
Unlike C-terminal and midregion PTH, which typically are grossly elevated in subjects with renal insufficiency, intact PTH assays are less influenced by the declining renal function[5].
PTH values are typically undetectable in hypocalcemia due to total hypoparathyroidism, but are found within the normal range in hypocalcemia due to partial loss or inhibition of parathyroid function.

4 PRINCIPLE OF THE TEST

The DRG Intact PTH Immunoassay is a two-site ELISA [Enzyme-Linked ImmunoSorbent Assay] for the measurement of the biologically intact 84 amino acid chain of PTH. Two different goat polyclonal antibodies to human PTH have been purified by affinity chromatography to be specific for well defined regions on the PTH molecule. One antibody is prepared to bind only the mid-region and C-terminal PTH 39-84 and this antibody is biotinylated. The other antibody is prepared to bind only the N-terminal PTH 1-34 and this antibody is labeled with horseradish peroxidase [HRP] for detection.

Streptavidin Well - Biotinylated Anti-PTH (39-84) --Intact PTH -- HRP conjugated Anti-PTH (1-34)

Although mid-region and C-terminal fragments are bound by the biotinylated anti-PTH (39-84), only the intact PTH 1-84 forms the sandwich complex necessary for detection. The capacity of the biotinylated antibody and the streptavidin coated microwell both have been adjusted to exhibit negligible interference by inactive fragments, even at very elevated levels.
In this assay, calibrators, controls, or patient samples are simultaneously incubated with the enzyme labeled antibody and a biotin coupled antibody in a streptavidin-coated microplate well.
At the end of the assay incubation, the microwell is washed to remove unbound components and the enzyme bound to the solid phase is incubated with the substrate, tetramethylbenzidine (TMB). An acidic stopping solution is then added to stop the reaction and converts the color to yellow. The intensity of the yellow color is directly proportional to the concentration of intact PTH in the sample. A dose response curve of absorbance unit vs. concentration is generated using results obtained from the calibrators. Concentrations of intact PTH present in the controls and patient samples are determined directly from this curve.

5 KIT COMPONENTS

Kit Components	Description	Quantity
RGT 1 = Reagent 1	Biotinylated PTH Antibody	1 x 7.0 mL
RGT 2 = Reagent 2	Peroxidase (Enzyme) labeled PTH Antibody	1 x 7.0 mL
RGT B = Reagent B	TMB Substrate [tetramethylbenzidine]	1 x 20 mL
RGT 3 = Reagent 3	Diluent [equine serum] for Patient Samples read off-scale	1 x 2 mL
RGT A = Reagent A	ELISA Wash Concentrate [Saline with surfactant]	1 x 30 mL
SOLN = Stopping Solution	ELISA Stop Solution [1 N sulfuric acid]	1 x 20 mL
RGT 4 = Reagent 4	Reconstitution Solution containing surfactant	1 x 5 mL
PLA = Microplates	One holder with Streptavidin Coated Strips.	12 x 8-well strips
CAL = Calibrators A: 0 pg/mL B –F: Refer to vial labels for exact concentrations	Lyophilized synthetic h-PTH. Lyophilized Zero calibrator [BSA solution with goat serum]. All other calibrators consist of synthetic h-PTH (1-84) in BSA solution with goat serum.	1 x 0.5 mL per level
CTRL = Controls 1 & 2 Refer to vial labels for exact concentrations	Lyophilized. 2 Levels. Synthetic h-PTH (1-84) in BSA solution with goat serum.	1 x 0.5 mL per level

5.1 MATERIAL AND EQUIPMENT REQUIRED BUT NOT PROVIDED

- Microplate reader.
- Microplate washer [if washer is unavailable, manual washing may be acceptable].
- Precision Pipettors to deliver 25, 100 and 150 µL.
- (Optional): A multi-channel dispenser or a repeating dispenser for 50, 100 and 150 µL.

6 WARNINGS AND PRECAUTIONS FOR USERS

Although the reagents provided in this kit has been specifically designed to contain no human blood components, the human patient samples, which might be positive for HBsAg, HBcAg or HIV antibodies, must be treated as potentially infectious biohazard. Common precautions in handling should be exercised, as applied to any untested patient sample. Stopping Solution consists of 1 N Sulfuric Acid. This is a strong acid. Although diluted, it still must be handled with care. It can cause burns and should be handled with gloves and eye protection, with appropriate protective clothing. Any spill should be wiped immediately with copious quantities of water. Do not breath vapor and avoid inhalation.

7 SAMPLE COLLECTION AND STORAGE

The determination of Intact PTH should be performed with EDTA plasma or serum.
EDTA plasma has been reported to demonstrate improved PTH stability as compared to serum[6].
To assay the specimen in duplicate, 50 µL of serum or EDTA plasma is required.
Collect whole blood without anticoagulant or lavender [EDTA] tube. After allowing blood to clot, the serum or plasma should be promptly separated, preferably in a refrigerated centrifuge, and stored at –20°C or lower.
Serum samples may be stored up to 8 hours at 2-8°C.
Serum samples frozen at –20°C are stable for up to 4 months.

8 REAGENT PREPARATION AND STORAGE

Store all kit components at 2-8 °C except Wash Concentrate and Stop Solution upon receipt prior to use

1. All reagents except the calibrators, kit controls and the Wash Concentrate are ready-to-use. Store all reagents at 2-8°C, except the Wash Concentrate, which should be kept at room temperature until dilution to avoid precipitation.
2. For each of the **calibrators** (Calibrator A through F) and **kit controls** 1 and 2, reconstitute each vial with 500 µL of Reagent 4 (Reconstitution Solution) and mix. Allow the vial to stand for 10 minutes and then mix thoroughly by gentle inversion to insure complete reconstitution. **Use the calibrators and controls as soon as possible upon reconstitution. Freeze (-20°C) the remaining calibrators and controls as soon as possible after use.**
   Standards and controls are stable at –20°C for 6 weeks after reconstitution with up to 3 freeze thaw cycles when handled as recommended in "Procedural Notes" section.
3. Reagent A: **Wash Concentrate**; Mix contents of wash concentrate thoroughly. If precipitate is present in the Wash Concentrate due to storage at lower temperature such as 4°C, dissolve by placing the vial in a 37°C water bath or oven with swirling or stirring. Add wash concentrate (30 mL) to 570 mL of distilled or deionized water and mix.
   The diluted working wash solution is stable for 90 days when stored at room temperature.

9 ASSAY PROCEDURE

1. Place sufficient **Streptavidin Coated Strips** in a holder to run all six (6) PTH calibrators, A - F of the Intact PTH CALIBRATORS [Exact concentration is stated on the vial label], Quality Control Sera and patient samples.
2. Pipet **25 µL** of sample into the designated or mapped well. ***Freeze (-20°C) the remaining calibrators and controls as soon as possible after use.***
3. Add or dispense **50 µL** of Reagent 1 (Biotinylated Antibody) into each of the wells which already contain the sample.
4. Add or dispense **50 µL** of Reagent 2 (Enzyme Labeled Antibody) into each of the same wells.
   Cover the microplate(s) with aluminum foil or a tray to avoid exposure to light, and place it on an **orbital shaker or rotator** set at 170 ± 10 rpm for **3 hours** ± 30 minutes at room temperature (22–28°C).

5. First aspirate the fluid completely and then wash/aspirate each well five (5) times with the Working Wash Solution (prepared from Reagent A), using an automatic microplate washer. The wash solution volume should be set to dispense 0.35 mL into each well.
6. Add or dispense **150 µL** of the Reagent B (TMB Substrate) into each of the wells.
7. With appropriate cover to avoid light exposure, place the microplate(s) on an **orbital shaker or rotator** set at 170 ± 10 rpm for **30 ±5 minutes** at room temperature (22–28°C).
8. Add or dispense **100 µL** of the Stopping Solution into each of the wells. Mix gently.
9. Read the absorbance of the solution in the wells within 10 minutes, using a microplate reader set to **450 nm** against 250 µL of distilled or deionized water. **Read** the plate **again** with the reader set to **405 nm** against distilled or deionized water.
   Note: The second reading is designed to extend the analytical validity of the calibration curve to the value represented by the highest calibrator, which is approximately 700 – 1,000 pg/mL. Hence, patient samples with PTH > 200 pg/mL can be quantified against a calibration curve consisting of the readings all the way up to the concentration equivalent to the highest calibrator using the 405 nm reading, away from the wavelength of maximum absorbance. In general, patient and control samples should be read using the 450 nm for PTH concentrations up to 200 pg/mL. PTH concentrations above 200 pg/mL should be interpolated using the 405 nm reading.
10. By using the final absorbance values obtained in the previous step, construct a calibration curve via cubic spline, 4 parameter logistics, or point-to-point interpolation to quantify the concentration of the intact PTH.

9.1 Procedural Notes

- Intact PTH 1-84 is a very labile molecule. Set up the assay immediately upon the reconstitution or the thawing of all calibrators, controls, and patient samples.
- It is recommended that all calibrators, controls, and patient samples are assayed in duplicate. The average absorbance units of duplicate sets should then be used for reduction of data and the calculation of results.
- The samples should be pipetted into the well with minimum amount of air-bubble. To achieve this, "reverse pipet" described in the package insert of the manufacturers of Pipettors is recommended.
- Patient samples with values greater than the highest calibrator (Calibrator F), which is approximately 700 – 1,000 pg/mL (see exact concentration on vial label), may be diluted with Reagent 3 (Sample Diluent) and reassayed. Multiply the result by the dilution factor.
- Reagents from different lot numbers must not be interchanged.
- If preferred, mix in equal volumes, in sufficient quantities for the assay, Reagent 1 (Biotinylated Antibody) and Reagent 2 (Enzyme Labeled Antibody) in a clean amber bottle, Then use 100 µL of the mixed antibody into each well. This alternative method should replace Step (3) and (4), to be followed with the incubation with orbital shaker.

10 CALCULATION OF RESULTS

10.1 Manual Method

1. For the 450 nm readings, construct a dose response curve (calibration curve) using the first five calibrators provided, i.e. Calibrators A, B, C, D and E. For the 405 nm readings, construct a second dose response curve using the three calibrators with the highest concentrations,
   i.e. Calibrators D, E and F.

2. Assign the concentration for each calibrator stated on the vial in pg/mL. Plot the data from the calibration curve on linear graph paper with the concentration on the X-axis and the corresponding A.U. on the Y-axis.
3. Draw a straight line between 2 adjacent points. This mathematical algorithm is commonly known as the "point-to-point" calculation. Obtain the concentration of the sample by locating the absorbance unit on the Y-axis and finding the corresponding concentration value on the X-axis. Patient and control samples should be read using the 450 nm for PTH concentrations up to 200 pg/mL. PTH concentrations above 200 pg/mL should be interpolated using the 405 nm reading.

10.2 Automated Method

Computer programs using cubic spline or 4 PL [4 Parameter Logistics] can generally give a good fit.

Sample Data *at 450 nm* [raw A.U. readout against distilled or deionized water]

Microplate Well	1st Reading Absorbance Unit	2nd Reading Absorbance Unit	Average Absorbance Unit	Intact PTH pg/mL	Intact PTH pg/mL – Result to report
Calibrator A	0.020	0.016	0.018		0
Calibrator B	0.056	0.051	0.054		7
Calibrator C	0.124	0.119	0.122		18
Calibrator D	0.388	0.393	0.391		55
Calibrator E	1.335	1.340	1.338		210
Control 1	0.200	0.200	0.200	27.6	27.6
Control 2	0.804	0.794	0.799	119	119
Patient Sample 1	0.147	0.136	0.142	19.1	19.1
Patient Sample 2	0.407	0.409	0.408	58.5	58.5
Patient Sample 3	2.375	2.454	2.415	> 200	*
Patient Sample 4	3.725	3.725	3.725	> 200	*

* Because the concentration readout is > 200 pg/mL, it is recommended to use the data obtained at 405 nm as shown in **Sample Data** *at 405 nm* in the table below.

Sample Data *at 405 nm* [raw A.U. readout against distilled or deionized water]

Microplate Well	1st Reading Absorbance Unit	2nd Reading Absorbance Unit	Average Absorbance Unit	Intact PTH pg/mL	Intact PTH pg/mL –Result to report
Calibrator A	0.014	0.008	0.011		0
Calibrator D	0.124	0.128	0.126		55
Calibrator E	0.428	0.425	0.427		210
Calibrator F	1.309	1.317	1.313		700
Control 1	0.074	0.066	0.070	< 200	¶
Control 2	0.260	0.251	0.256	121	π
Patient Sample 1	0.049	0.043	0.046	< 200	¶
Patient Sample 2	0.132	0.133	0.133	< 200	¶
Patient Sample 3	0.758	0.782	0.770	401	401
Patient Sample 4	1.314	1.321	1.318	> 700	⇐

¶ For samples with readout < 200 pg/mL, it is recommended to use the data obtained at 450 nm as shown in **Sample Data** *at 450 nm* in the table above. This practice should give the results with optimum sensitivity of the assay.

π Although the readout for Control (2) < 200 pg/mL, it is recommended that the actual result be read out and recorded for quality control evaluation purposes. Further, absorbance for Control 2 is sufficiently high to be analytically valid.

⇐ The absorbance readout is off-scale or higher than the average absorbance of the highest calibrator. Sample should be repeated with dilution.

*NOTE: The data presented are **for illustration purposes only** and must not be used in place of data generated at the time of the assay.*

11 QUALITY CONTROL

Control serum or serum pools should be analyzed with each run of calibrators and patient samples. Results generated from the analysis of the control samples should be evaluated for acceptability using appropriate statistical methods. In assays in which one or more of the quality control sample values lie outside the acceptable limits, the results for the patient sample may not be valid.

12 LIMITATIONS OF PROCEDURE

The DRG PTH ELISA kit has exhibited no "high dose hook effect" with samples spiked with 2,100,000 pg/mL of Intact PTH.
Samples with intact PTH levels greater than the highest calibrator, however, should be diluted and reassayed for correct values.
Like any analyte used as a diagnostic adjunct, intact PTH results must be interpreted carefully with the overall clinical presentations and other supportive diagnostic tests.

13 EXPECTED VALUES

Intact PTH levels were measured in 148 apparently normal individuals in the U.S. with the Intact PTH ELISA.
The values obtained ranged from 9.0 to 94 pg/mL for serum.
Based on statistical tests on skewness and kurtosis, the population, when transformed logarithmically, follows the normal or Gaussian distribution.
The geometric mean + 2 standard deviations of the mean were calculated to be 10.4 to 66.5 pg/mL for serum.

14 PERFORMANCE CHARACTERISTICS

14.1 Accuracy

Three hundred and nine (309) patient samples, with intact PTH values ranging from 1.0 to 833 pg/mL were assayed by the previous DRG PTH kit and the updated DRG PTH kit. Linear regression analysis gives the following statistics:

DRG ELISA = 1.06 - 1.49 pg/mL r = 0.998 N = 309

14.2 Sensitivity

The sensitivity, or minimum detection limit, of this assay is defined as the smallest single value, which can be distinguished from zero at the 95% confidence limit.
The DRG PTH ELISA has a calculated sensitivity of 1.57 pg/mL.

14.3 Specificity and Cross-Reactivity

The antibodies used in the DRG PTH ELISA were purified by affinity chromatography to be specific for well-defined regions on the PTH molecule. The peroxidase labeled antibody recognizes only the N-terminal region or the 1-34 amino acid sequence of the PTH molecule; whereas the biotinylated antibody is specific to the 39-84 segment. Accordingly, only intact PTH, which requires binding by both the enzyme conjugated and biotinylated antibodies, can be detected by this assay.
To further achieve the specificity of this assay, conjugation and biotinylation and the molar ratios thereof, have been optimized to minimize detection of fragments of PTH. Human PTH 1-34 at levels up to 300 pg/mL and the C-terminal 39-84 fragment at levels up to 300,000 pg/mL give molar cross reactivites to PTH of less than 2% and 0.02%, respectively.

14.4 Precision and Reproducibility

The precision (intra-assay variation) of the DRG PTH ELISA Test was calculated from 25 replicate determinations on each of the two samples.

Intra-Assay Variation

Sample	Mean Value (pg/mL)	N	Coefficient of variation %
A	32.4	25	6.08
B	178.2	25	3.68

The total precision (inter-assay variation) of the DRG PTH ELISA Test was calculated from data on two samples obtained in 21 different assays, by three technicians on three different lots of reagents.

Inter-Assay Variation

Sample	Mean Value (pg/mL)	N	Coefficient of Variation %
A	30.3	21	3.6
B	159.1	21	2.8

14.5 Recovery

Various amounts of PTH 1-84 were added to three different patient sera to determine the recovery. The results are described in the following table:

Serum Sample	PTH Endogenous (pg/ml)	PTH added (pg/ml)	Expected Value (pg/ml)	Measured Value (pg/ml)	Recovery (%)
A	32.7	132	165	168	102%
	20.6	264	285	288	101%
	13.5	396	410	413	101%
B	68.6	132	201	191	95%
	51.7	264	316	344	109%
	45.0	396	441	462	105%
C	19.9	132	152	165	109%
	15.4	264	279	275	99%
	13.3	396	409	424	104%

Average 103%

14.6 Linearity of Patient Sample Dilutions: Parallelism

Four patient serum samples were diluted with Reagent 4 (the Diluent for Patient Samples read off-scale). Results in pg/mL are shown below:

Sample	Dilution	Expected	Observed	% Observed ÷ Expected
A	Undiluted	-	322	-
	1:2	161	148	92%
	1:4	80.5	73.1	91%
	1:8	40.3	41.5	103%
B	Undiluted	-	230	-
	1:2	115	97	84%
	1:4	58	55	95%
	1:8	29	30	103%
C	Undiluted	-	176	-
	1:2	88	82	93%
	1:4	44	45	102%
	1:8	22	24	109%
D	Undiluted	-	426	-
	1:2	213	192	90%
	1:4	107	90	84%
	1:8	53	47	89%

Average 95%

1 REFERENCES / LITERATURE / LITERATUR / BIBLIOGRAFIA

1. Segre, G.V., Niall H.D., Habener J.F. et. al. : Metabolism of parathyroid hormone: physiological and clinical significance. Am. J. Med. 56: 774,1974.
2. Mallete, L.E., Gagel, R.F.: Parathyroid Hormone and Calcitonin. In: Murray J.F. (ed) Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. American Society for Bone and Mineral Research, Kelseyville; William Byrd Press, Richmond, pp. 65-69, 1990.
3. Bilezikian, J.P.: Primary Hyperparathyroidism. In: Murray J.F. (ed) Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. American Society for Bone and Mineral Research, Kelseyville; William Byrd Press, Richmond, pp. 109-111, 1990.
4. Stewart, A.F.: Humoral Hypercalcemia of Malignancy. In: Murray J.F. (ed) Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism. American Society for Bone and Mineral Research, Kelseyville; William Byrd Press, Richmond, pp. 115-118, 1990.
5. Mallette, L.E.: The parathyroid polyhormones: New concepts in the spectrum of peptide hormone action. Endocrin. Rev. 12:110-117, 1991.
6. Kruger, L.., Rosenblum, S., Zaazra, J. and Wong, J. Intact PTH is stable in unfrozen EDTA plasma for 48 hours prior to laboratory Analysis. Clin. Chem. 41:6: page S47, 1995.

Further Suggested Reading:

1. Raisz, L.G., Yajnik, C.H., Bockman, R.S., and Bower, B.B.: Comparison of commercially available parathyroid hormone immunoassay in the differential diagnosis of hypercalcemia due to primary hyperparathyroidism or malignancy. Ann. Intern. Med. 91:739-740, 1979.
2. Endres, D., Brickman, A., Goodman, W., Maloney, D., and Sherrard, D.: N-Terminal PTH radioimmunoassays in assessment of renal osteodystrophy. Kidney International. 21:132, 1982.
3. Dambacher, M.A., Fischer, J.A., Hunziker, W.H., et.al.: Distribution of circulating immunoreactive components of parathyroid hormone in normal subjects and in patients with primary and secondary hyperparathyroidism: the role of kidney and of the serum calcium concentration.,Clin. Sci. 57:435,1979.
4. Kao, P.C., Jiang, N.S., Klee, G.G., and Purnell, D.C.: Development and validation of a new radioimmunoassay for parathyrin (PTH).,Clin. Chem. 28:69, 1982.
5. Endres, D.B., Villanueva, R., Sharp, C.F. Jr, Singer, F.R.: Measurement of parathyroid hormone.,Endocrinol Metab. Clin. North Am. 18:611-629,1989.
6. Kao, P.C., van Heerden, J.A., Grant, C.S., Klee, G.G., Khosla S: Clinical performance of parathyroid hormone immunometric assays.,Mayo Clin. Proc. 67:637-645, 1992.
7. Marcus, R.: Laboratory diagnosis of primary hyperparathyroidism.,Endocrinol Metab. Clin. North Am. 18:647-658, 1989.

Appendix G: Dependent Variable Percent Change Tables

Table 20. Total Body aBMD and Body Composition %Δ (means ± SD).

	Time	ROTC (n=18)	Controls (n=18)
Total Body aBMD (g/cm^2)	Pre-Mid	-0.24 ± 2.23	-0.75 ± 1.32
	Mid-Post	-1.16 ± 4.62	0.35 ± 1.20
	Pre-Post	-1.35 ± 3.82	-0.39 ± 1.01
Total Body BMC (g)	Pre-Mid	0.20 ± 1.42	0.04 ± 1.53
	Mid-Post	0.38 ± 1.06	-0.05 ± 1.08
	Pre-Post	0.59 ± 0.95	0.00 ± 1.55
Total Body % Fat	Pre-Mid	-2.99 ± 6.21	-2.68 ± 5.88
	Mid-Post	0.10 ± 7.77	0.57 ± 5.91
	Pre-Post	-2.82 ± 9.27	-2.03 ± 7.70
Total Body Fat Mass (kg)	Pre-Mid	-2.67 ± 6.69	-2.66 ± 7.16
	Mid-Post	0.05 ± 7.96	0.42 ± 7.02
	Pre-Post	-2.68 ± 11.25	-2.14 ± 9.52
Total Body BFLBM (kg)	Pre-Mid	0.87 ± 2.21	0.97 ± 1.43
	Mid-Post	-0.20 ± 2.55	-0.39 ± 2.56
	Pre-Post	0.69 ± 2.46	0.58 ± 2.80

aBMD: Areal Bone Mineral Density (g/cm^2)
BMC: Bone Mineral Content (g)
BFLBM: Bone Free Lean Body Mass (kg)

Table 21. Regional aBMD and Body Composition %Δ (means ± SD).

	Time	ROTC (n=18)	Controls (n=18)
Arms BMC (g)	Pre-Mid	0.47 ± 2.96	0.34 ± 2.83
	Mid-Post	-0.31 ± 2.36	-0.65 ± 2.89
	Pre-Post	0.22 ± 1.74	-0.25 ± 2.41
Arms % Fat	Pre-Mid	-3.41 ± 7.02	-3.12 ± 6.31
	Mid-Post	-1.20 ± 9.87	-0.70 ± 8.05
	Pre-Post	-4.32 ± 9.19	-3.75 ± 9.69
Arms Fat Mass (kg)	Pre-Mid	-1.82 ± 7.92	-2.73 ± 6.71
	Mid-Post	-2.98 ± 11.53	-1.96 ± 9.80
	Pre-Post	-4.63 ± 12.71	-4.61 ± 11.05
Arms BFLBM (kg)	Pre-Mid	2.36 ± 3.89	1.29 ± 2.87
	Mid-Post	-1.89 ± 3.65	-1.17 ± 3.30
	Pre-Post	0.58 ± 3.68	0.17 ± 3.70
Legs BMC (g)	Pre-Mid	0.03 ± 1.57	0.74 ± 14.51
	Mid-Post	-0.01 ± 1.50	1.77 ± 5.57
	Pre-Post	0.03 ± 1.25	2.93 ± 12.01
Legs % Fat	Pre-Mid	-2.94 ± 5.06	-6.31 ± 15.99
	Mid-Post	-0.92 ± 8.06	4.10 ± 8.79
	Pre-Post	-3.80 ± 8.68	-0.86 ± 7.13
Legs Fat Mass (kg)	Pre-Mid	-2.45 ± 5.20	-4.39 ± 7.81
	Mid-Post	-0.27 ± 8.61	2.95 ± 6.81
	Pre-Post	-2.71 ± 10.08	-1.17 ± 9.10
Legs BFLBM (kg)	Pre-Mid	1.21 ± 2.92	5.22 ± 13.48
	Mid-Post	0.88 ± 3.51	-2.93 ± 9.71
	Pre-Post	2.09 ± 4.27	3.02 ± 12.06

BMC: Bone Mineral Content (g)

BFLBM: Bone Free Lean Body Mass (kg)

Table 22. Lumbar Spine and Dual Hip aBMD (g/cm^2) %Δ (means ± SD).

	Time	ROTC (n=18)	Controls (n=18)
Lumbar Spine 1-4	Pre-Post	0.52 ± 1.33#	-0.47 ± 1.60
Dominant			
Femoral Neck	Pre-Post	0.76 ± 1.31#	-1.02 ± 1.72
Trochanter	Pre-Post	0.32 ± 1.77#	-1.02 ± 2.17
Total Hip	Pre-Post	0.63 ± 0.93#	-0.26 ± 1.18
Non-Dominant			
Femoral Neck	Pre-Post	0.01 ± 1.88	0.13 ± 1.67
Trochanter	Pre-Post	0.59 ± 2.00	-1.03 ± 4.98
Total Hip	Pre-Post	0.36 ± 1.07	0.14 ± 1.37

Significantly different than Controls $p \leq 0.05$

Table 23. Lumbar Spine and Dual Hip BMC (g) %Δ (means ± SD).

	Time	ROTC (n=18)	Controls (n=18)
Lumbar Spine 1-4	Pre-Post	2.13 ± 7.62	0.30 ± 1.56
Dominant			
Femoral Neck	Pre-Post	0.31 ± 1.72	-1.06 ± 2.37
Trochanter	Pre-Post	0.86 ± 3.37	-2.18 ± 4.15
Total Hip	Pre-Post	0.47 ± 1.29	-0.96 ± 1.56#
Non-Dominant			
Femoral Neck	Pre-Post	0.56 ± 2.25	0.09 ± 1.39
Trochanter	Pre-Post	1.01 ± 5.77	0.82 ± 3.82
Total Hip	Pre-Post	0.38 ± 1.78	0.16 ± 1.16

Significantly different than ROTC ($p \leq 0.050$)

Table 24. Hip Structural Analysis Variables %Δ (means ± SD).

	Time	ROTC (n=18)	Controls (n=18)
Dominant Hip			
Strength Index	Pre-Post	-4.50 ± 13.98	-0.06 ± 12.77
Buckling Ratio	Pre-Post	-25.53 ± 64.13	1.49 ± 39.84
Section Modulus (mm^3)	Pre-Post	-1.50 ± 6.08	-1.07 ± 5.91
CSMI (mm^4)	Pre-Post	-2.09 ± 5.86	-0.31 ± 4.69
Non-Dominant			
Strength Index	Pre-Post	-3.54 ± 15.40	0.04 ± 12.92
Buckling Ratio	Pre-Post	-15.57 ± 40.95	-11.99 ± 57.77
Section Modulus (mm^3)	Pre-Post	-0.20 ± 3.94	-0.37 ± 3.63
CSMI (mm^4)	Pre-Post	1.59 ± 4.58	-0.71 ± 5.11

CSMI: Cross-Section Moment of Inertia

Table 25. 4% Non-Dominant Tibia pQCT Variables %Δ (means ± SD).

	Time	ROTC (n=18)	Controls (n=18)
Total			
BMC (mg/mm)	Pre-Post	-0.05 ± 0.94	0.01 ± 1.02
vBMD (mg/cm^3)	Pre-Post	0.12 ± 1.15	-0.37 ± 1.68
Area (mm^2)	Pre-Post	-0.19 ± 1.62	0.37 ± 1.55
	Pre-Post	0.07 ± 1.36	-0.36 ± 2.26
Trabecular			
BMC (mg/mm)	Pre-Post	-0.28 ± 2.00	0.31 ± 1.50
vBMD (mg/cm^3)	Pre-Post	0.01 ± 0.72	-0.30 ± 1.40
Area (mm^2)	Pre-Post	-0.29 ± 2.08	0.60 ± 1.88
BSI (mg^2/mm^4)	Pre-Post	-0.27 ± 2.18	0.01 ± 2.20
Periosteal Circ. (mm)	Pre-Post	-0.09 ± 0.81	0.19 ± 0.78

BMC: Bone Mineral Content
vBMD: Volumetric Bone Mineral Density
BSI: Bone Strength Index
Circ: Circumference

Table 26. 38% Non-Dominant Tibia pQCT Variables %Δ (means ± SD).

	Time	ROTC (n=18)	Controls (n=18)
Total			
BMC (mg/mm)	Pre-Post	0.30 ± 0.82	0.04 ± 0.47
vBMD (mg/cm³)	Pre-Post	0.14 ± 0.37	0.02 ± 0.33
Area (mm²)	Pre-Post	0.16 ± 0.59	0.02 ± 0.50
Cortical			
BMC (mg/mm)	Pre-Post	0.25 ± 0.83	0.04 ± 0.52
vBMD (mg/cm³)	Pre-Post	0.02 ± 0.39	-0.02 ± 0.36
Area (mm²)	Pre-Post	0.23 ± 1.10	0.05 ± 0.72
Thickness (mm)	Pre-Post	0.18 ± 1.08	0.05 ± 0.70
Periosteal Circ. (mm)	Pre-Post	0.08 ± 0.29	0.01 ± 0.25
Endosteal Circ. (mm)	Pre-Post	0.02 ± 0.56	-0.01 ± 0.58
iPolar (mm^4)	Pre-Post	0.39 ± 1.19	0.06 ± 0.71
SSI (mm³)	Pre-Post	0.08 ± 1.06	0.58 ± 1.45

BMC: Bone Mineral Content
vBMD: Volumetric Bone Mineral Density
Circ: Circumference
SSI: Stress Strain Index

Table 27. 66% Non-Dominant Tibia pQCT Variables %Δ (means ± SD).

	Time	ROTC (n=18)	Controls (n=18)
Total			
BMC (mg/mm)	Pre-Post	0.23 ± 0.56	0.13 ± 0.30
vBMD (mg/cm³)	Pre-Post	-2.51 ± 12.31	0.15 ± 0.63
Area (mm²)	Pre-Post	1.71 ± 8.40	-0.02 ± 0.67
Cortical			
BMC (mg/mm)	Pre-Post	0.06 ± 1.26	0.17 ± 0.46
vBMD (mg/cm³)	Pre-Post	-0.01 ± 0.81	0.06 ± 0.48
Area (mm²)	Pre-Post	0.07 ± 0.87	0.11 ± 0.44
Thickness (mm)	Pre-Post	-2.35 ± 11.66	0.14 ± 0.65
Periosteal Circ. (mm)	Pre-Post	0.96 ± 4.64	-0.01 ± 0.34
Endosteal Circ. (mm)	Pre-Post	2.39 ± 11.86	-0.05 ± 0.75
iPolar (mm^4)	Pre-Post	2.55 ± 10.77	0.07 ± 0.79
SSI (mm³)	Pre-Post	1.80 ± 8.41	-0.13 ± 1.19
Muscle CSA (mm²)	Pre-Post	2.58 ± 2.28	0.76 ± 3.25

BMC: Bone Mineral Content
vBMD: Volumetric Bone Mineral Density
Circ: Circumference
SSI: Stress Strain Index

Table 28. Muscular Strength and Power Measures %Δ (means ± SD).

	Time	ROTC (n=18)	Controls (n=18)
Right Hand Grip (kg)	Pre-Mid	-0.14 ± 7.51	0.73 ± 9.59
	Mid-Post	-4.46 ± 9.99	-3.06 ± 6.56
	Pre-Post	-4.23 ± 8.73	-2.11 ± 9.66
Left Hand Grip (kg)	Pre-Mid	-0.22 ± 9.12	2.06 ± 7.92
	Mid-Post	-5.57 ± 10.23	-6.14 ± 11.85
	Pre-Post	-5.14 ± 7.32	-3.72 ± 12.78
Jump Height (in)	Pre-Mid	3.91 ± 5.48	4.84 ± 6.08
	Mid-Post	-2.46 ± 6.89	-2.38 ± 4.04
	Pre-Post	2.38 ± 4.64	2.66 ± 5.94
Time in Air (sec)	Pre-Mid	2.63 ± 3.93	2.53 ± 3.35
	Mid-Post	-1.17 ± 3.52	-0.74 ± 2.85
	Pre-Post	1.86 ± 3.87	1.80 ± 3.47
Jump Power (w)	Pre-Mid	2.16 ± 3.92	4.99 6.42
	Mid-Post	-2.44 ± 6.16	-6.03 ± 6.92
	Pre-Post	-0.63 ± 5.56	-0.25 ± 6.82
Jump Velocity (m/s)	Pre-Mid	2.06 ± 3.77	3.49 ± 5.73
	Mid-Post	-3.05 ± 5.87	-4.71 ± 7.34
	Pre-Post	-1.39 ± 6.63	-1.14 ± 5.47
Leg Press (kg)	Pre-Mid	8.83 ± 10.28	9.59 ± 11.49
	Mid-Post	1.98 ± 8.89	0.24 ± 7.49
	Pre-Post	10.92 ± 13.08	10.06 ± 12.89
Bench Press (kg)	Pre-Mid	3.95 ± 5.04	3.17 ± 4.42
	Mid-Post	-0.18 ± 5.12	1.41 ± 8.91
	Pre-Post	3.81 ± 6.86	4.46 ± 9.57

Table 29. Aerobic Capacity Testing Measures %Δ (means ± SD).

	Time	ROTC (n=18)	Controls (n=18)
Absolute VO_2 Peak (L/min)	Pre-Post	2.64 ± 6.01	1.19 ± 4.32
Relative VO_2 Peak (kg/mL/min)	Pre-Post	2.56 ± 4.80	1.41 ± 4.27
Respiratory Exchange Ratio	Pre-Post	-1.27 ± 3.33	-3.99 ± 5.58
Maximum Heart Rate (bpm)	Pre-Post	0.30 ± 2.09	0.52 ± 2.67
RPE	Pre-Post	-0.11 ± 5.99	1.82 ± 6.98
Time to Exhaustion (min)	Pre-Post	7.79 ± 8.85	5.05 ± 12.85

RPE: Rating of Perceived Exertion

Appendix H: Sclerostin Scatterplots

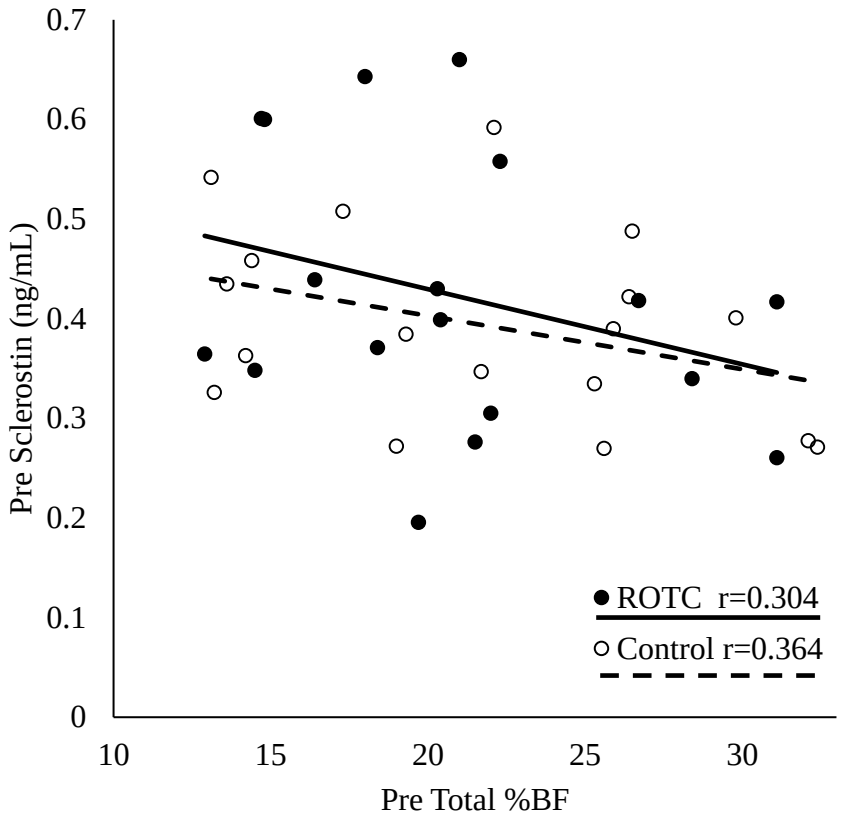


Figure 12. Correlation Between Baseline Sclerostin and Total % Body Fat.

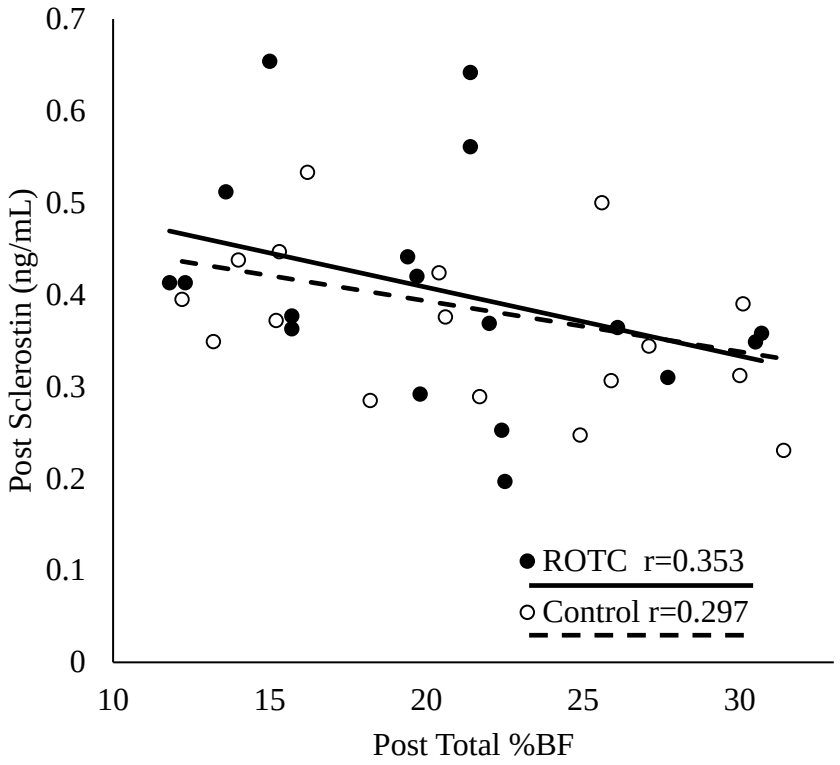


Figure 13. Correlation Between Post Sclerostin and Total % Body Fat.

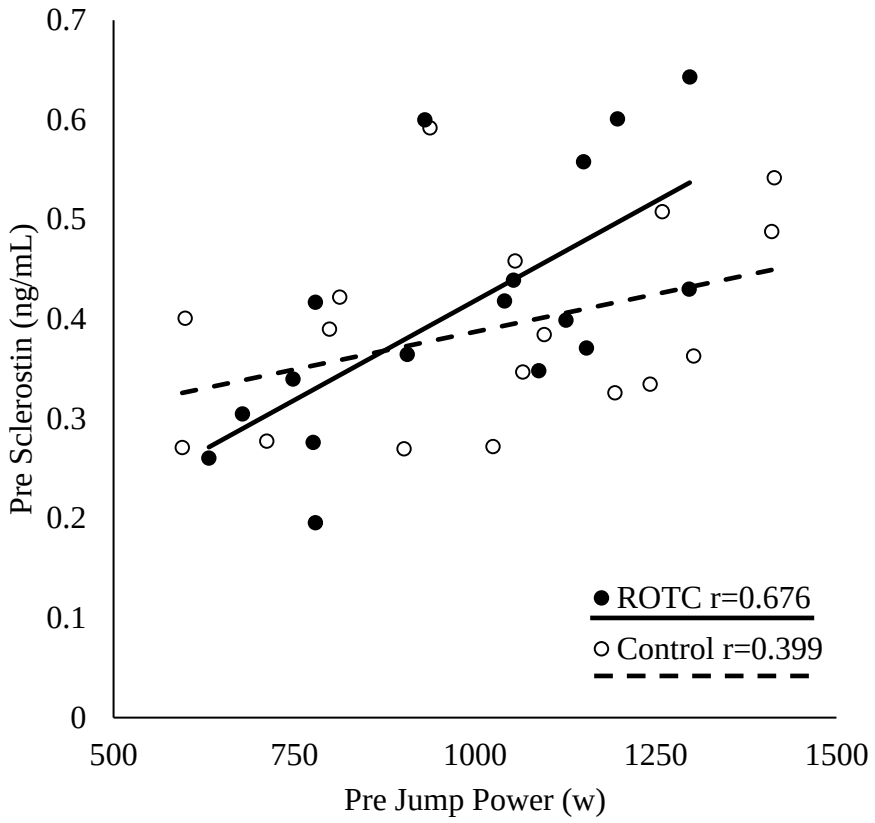


Figure 14. Correlation Between Baseline Sclerostin and Jump Power.

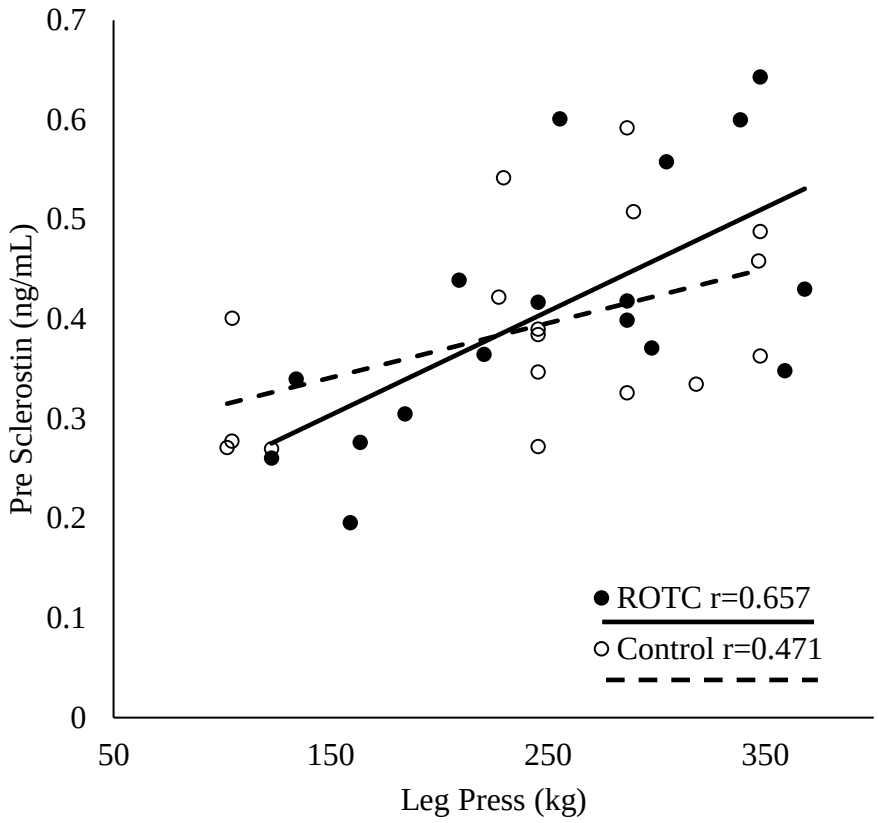


Figure 15. Correlation Between Baseline Sclerostin and Leg Press

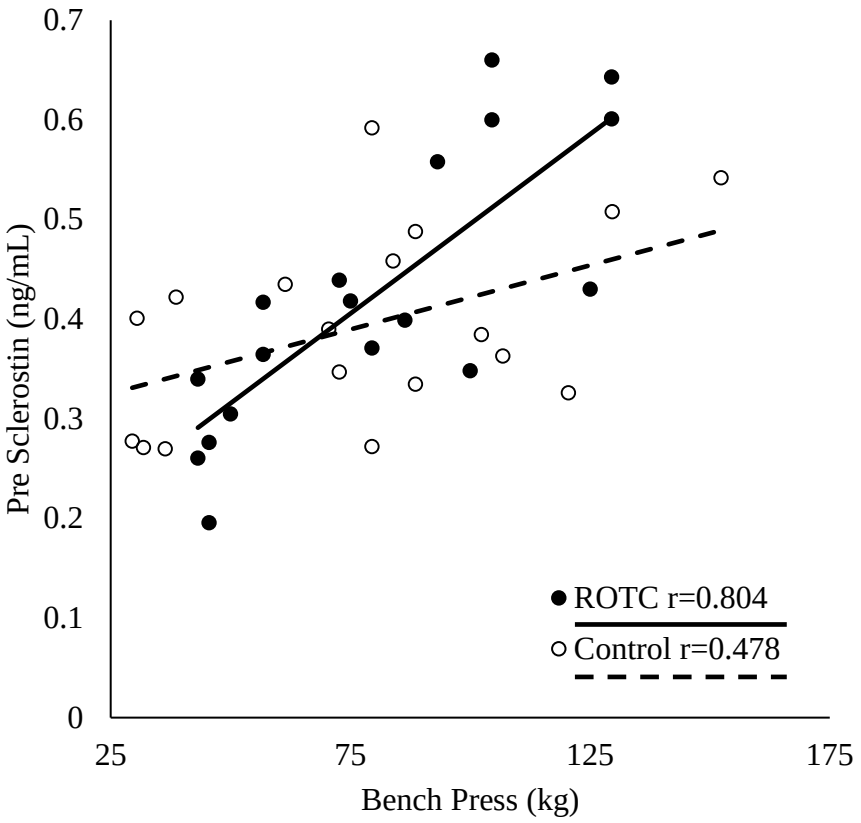


Figure 16. Correlation Between Baseline Sclerostin and Bench Press