THE EFFECTS OF LEAN MASS AND HORMONAL CHARACTERISTICS ON BONE GEOMETRY AND BONE STRENGTH IN YOUNG ADULT WOMEN

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DEDICATION

This thesis is dedicated to my husband, Chris, and my family, Myron, Carol, Pat and Katie. Without their enduring love and support, this thesis would not have been possible. They provided me with the encouragement and confidence to continue my education, and for that, I am forever grateful.

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TERMINOLOGY

Areal bone mineral density (aBMD) - the total grams of bone mineral per unit (g/cm^2) of bone area commonly assessed by dual energy x-ray Absorptiometry (also referred to as Bone Mineral Density).

Bending--when force is applied to the bone, the convex surface of the bone experiences tension and the concave surface experiences compression [18]. Example: Forearm during a bicep curl.

Bone--a complex system composed of hard connective tissue consisting of cells affixed in a matrix of organic (type I collagen), inorganic (mainly hydorxyapatite crystals composed of calcium and phosphate), and water.

Bone Geometry—property of bone that refers to its mineralized tissue configuration, placing great importance on the amount of tissue and their distribution throughout the particular bone.

Bone growth—enlargement of the skeleton through cellular activity and environmental strain (e.g. mechanical loading and muscle activity).

Bone mineral content (BMC)--the total grams (g) of bone mineral within the scanned region. A major determinant of the material properties of bone. Directly associated with bone strength and stiffness, accounting for more than 80% of the strength component (also referred to as Bone Mass).

Bone Modeling—process in which bone grows and becomes stronger through an organized bone cell activity of osteoblasts and osteoclasts. Increases bone strength by increasing or adding mass and improving the existing geometry.

Bone Stiffness—refers to a bone's elastic properties. Physiologically important because it describes a bone's ability to withstand a load, accommodate the load, then return to its original shape.

Bone Strength—"ultimate load" bone can withstand until failure. Intrinsic quality or property that is independent of size.

Bone Turnover—blanket term used to describe the adding or subtracting bone via growth, modeling and remodeling.

Compression Stress—stress produced when two forces are aimed toward each other along a straight line [18]. Causes the bone to both shorten and widen in order to absorb maximal stress. Example: Hip joint during walking.

Cortical Bone—the superficial thin layer of bone, forming the external portion of long bones and consisting mainly of dense, calcifies tissue.

Cross-sectional Moment of Inertia (CSMI)—property of bone that refers to its resistance to bending.

Dual Energy X-ray Absorptiometry (DXA)—bone measurement modality that uses two contrasting x-ray beams to yield a two dimensional representation of the skeleton. DXA calculates the attenuation values of photons that pass from the x-ray tube through the measurement site of interest. Outcome variables include bone mineral content (BMC) and areal bone mineral density (aBMD).

Endosteum—inner surface of cortical bone that faces bone marrow.

High-impact loading—characterized by both high rate and high magnitude loadings. Evidence suggests that loadings that include strain at a high rate and high peak force in diverse movements may be the most effective in enhancing bone formation, especially in

girls/women. Recent data suggests programs that incorporate high-impact loadings are effective in maintaining and improving bone mass and preventing bone loss due to age.

High-magnitude loading—characterized by high peak forces of loading. May have a greater influence on bone mass versus large numbers of loading cycles. Bone response can be achieved with high magnitude and a low number of loading cycles.

Hip Structural Analysis (HSA)—A predictive computer algorithm used to estimate bone cross-sectional geometry.

Lamellar bone—the normal type of adult bone. Replaces immature woven by arranging its collagen in a repeating fashion along the lines of force. Resembles multi-layer plywood.

Low-magnitude loading—characterized by low peak forces of loading. When low, the number of loading cycles may become an important factor to maintain bone structure.

Macromodeling—process in which cells and collagen are organized. Mainly responsible for the size, shape, and strength of bones.

Mechanical Loading-refers to the applied forces placed on the skeleton or individual bones via forms of physical movement or activity.

Micromodeling—process in which cells and collagen are organized. Responsible for determining what kind of tissue is to be formed. Even after growth stops this process continues.

Odd-impact loading-- charactertized by rapid accelerating and decelerating movements with a high magnitude of non-conventional movements of the body and hips.

Osteoblasts—cell formation responsible for bone formation. Produce a bone matrix composed of collagen and other substances that ultimately becomes calcified.

Osteoclasts—a multinucleated bone cell responsible for the removal of bone (resorption).

Peripheral Quantitative Computed Tomography (pQCT)—QCT-type bone measurement modality that provides a three-dimensional representation of a particular site of interest. Measures the attenuation of radiation as it passes from the source to the site of interest. Outcome measures include BMC, BMD, bone and muscle cross-sectional area, bone strength, and unlike DXA, has the ability to differentiate between types of bone and may be more sensitive to changes in bone due to physical activity.

Periosteum—outer surface of cortical bone facing tissue and muscle. Bone appositional site during growth and development.

Remodeling—process in which old bone is removed and new bone is formed at the same site at variable time intervals.

Repetitive loading—characterized by continuous loading patterns for an extended duration. Some exercise studies have shown repetitive loading (e.g. walking) can increase bone mass, however others have found no significance.

Repetitive non-impact loading—loading modality characterized by the ability to elicit great numbers of musculoskeletal movements, however lacking ground or surface impacts.

Resorption—process in which bone is removed via osteoclast activity.

Shear stress—stress produced when two forces are aimed parallel to one another but not along the same line [18]. Example: Stopping quickly on a soccer field. The foot is stationary, however body mass is still motion, placing stress on the tibia.

Strain—describes the deformation of bone from its original shape under certain loading conditions. It is equal to the change in length divided by the original.

Strain cycles—refers to the number of loading bouts. Less important than magnitude, however there are also a certain number of cycles to maintain bone structure. May be more important if magnitude is low. Bone strength is increased if bouts are shorter and separated compared to long continuous bouts.

Strain distribution—refers to the placement of the strain on particular bone. Bone tends to adapt better if strains deviate from normal loading patterns.

Strain magnitude—magnitude or size of the load placed on the bone during a particular bout. There is a Minimum Effective Strain (MES) to maintain bone structure (200-2500μЄ).

Strain Rate—rate at which the load is applied. Proportional to the dynamic load magnitude.

Stress—refers to the intensity of the load applied. The force applied per unit area (classified as compressive, tensile, or shear). Obtained by dividing the force and the area of bone in which it is applied.

Tensile stress—stress produced when two forces are aimed away from each other along a straight line [18]. Example: The patella of the knee being pulled both by the quadriceps muscle and the patellar tendon at the same time.

Torsion—occurs when shear stress is experienced along the length of the bone.

Trabecular Bone—cancellous bone, forming the internal component of bone consisting of both horizontal and vertical plates.

Volumetric Bone Mineral Density (vBMD)—measure of the volumetric tissue density of appendicular bone.

Woven bone—immature bone characterized by random arrangement of collagen.

1

RATIONALE, SPECIFIC AIMS, AND HYPOTHESES

1 1 RATIONALE

1.1.1 The relationship between mechanical loads and estrogen on bone strength in humans is not well documented

Although skeletal development is globally determined by genetic factors early in life [1], hormonal status and mechanical loading are also important determinants of bone development, peak bone mass accrual, rates of bone turnover, as well as rate of bone loss prior to menopause [2-8]. Bone is thought to adapt its strength primarily to mechanical demands from growth, changes in muscle force and physical activity [9-11]. The effects of mechanical loading seem to primarily effect the periosteal growth of the loaded bones, increasing modeling and remodeling on the periosteum, which increases overall bone strength [12, 13]. Other factors, specifically sex steroids, may influence bone loss by mediating the impact of loading on bone by altering the sensitivity of the periosteal and/or endosteal surfaces to loading. Also, estrogen has been shown to inhibit periosteal expansion and stimulate endocortical contraction [14, 15], suggesting decrease sensitivity to everyday loads. Following cessation of menses either via primary or secondary amenorrhea, which creates an absence and/or lower levels of circulating estrogen, increased periosteal formation and endocortical expansion occur, suggesting decreased sensitivity to loading [16-22]. However, there are few data in humans that explore the interaction mechanical loading and sex steroids on bone mass and bone strength in premenopausal women.

1.1.2 Bone Geometry and Volumetric Trabecular BMD are Important Parameters to Measure In Mechanical Loading Models

Until recently, much of skeletal development research focused on the changes in bone density under a variety of environments and interventions. These changes in bone were mostly measured by DXA, the most widely used method to evaluate bone mass in clinical and research settings. As discussed in section 2.3.1, DXA is unable to assess bone structure or separate cortical and trabecular bone compartments which may respond differently to loading [23]. To supplement DXA outcomes, hip structure analysis (HSA) [23] has been used to

estimate proximal femur bone geometry and strength, but it to is limited by DXA's 2-dimensional technology. Peripheral quantitative computed tomography (pQCT) provides a means with which to directly evaluate cross-sectional geometry and volumetric bone mineral density at the trabecular and cortical regions of long bones (tibia, femur and radius), allowing us to estimate bone *strength* [24]. Mechanical loading can increase bone diameter with little or no change in bone mass which would show up as a decrease in aBMD measured by DXA. Also, trabecaular bone may be disproportionately affected by hormonal and nutritional factors which would not be apparent in DXA studies. **Therefore, in this study, we use pQCT to assess cortical and trabecular bone vBMD, bone geometry and estimates of bone strength.**

1.2 SPECIFIC AIMS AND HYPOTHESES

Specific Aim 1: To determine predictors of volumetric bone density, bone geometry and strength in healthy premenopausal women.

The *working hypothesis* for this aim, based on data presented in the background section, is that the indices of mechanical load (lean mass and muscle CSA) will be the primary predictor of bone strength in premenopausal women. Other factors, including sex steroids, menstrual cycle characteristics and nutrition will influence vBMD and geometry, especially in trabecular regions. However, loading will be the strongest predictor of cortical strength.

Specific Aim 2: To explore the relationships of sex steroids and mechanical loading on cortical and trabecular bone strength in healthy premenopausal women.

The *working hypothesis* for this aim, based on animal and human data is that premenopausal women with the highest levels of estrogen and highest loads will have the greatest bone *strength* due to greater bone area. *Volumetric* bone density and bone mineral content will also be greatest in women with both high levels of estrogen and highest loads on the bone,

while those with the lowest levels of estrogen and smallest loads on the bone will have the smaller measures of geometry, vBMD and strength.

2

LITERATURE REVIEW

2.1 INTRODUCTION

2.1.1 Osteoporosis is a significant public health problem

Osteoporosis, a disease related to low bone mass and increased risk of fracture, is a significant public health problem with debilitating effects on health and physical function. It is estimated that up to 60% of all women and 30% of all men will suffer from a fracture directly related to osteoporosis in their lifetime[25, 26]. In the United States, 26% of women aged ≥65 years and >50% of women aged ≥85 years have osteoporosis. Over 1.5 million fractures per year are attributable to osteoporosis, and these fractures result in 500,000 hospitalizations, 800,000 emergency room visits, 2.6 million physician visits, 180,000 nursing home placements, and $12 billion to $18 billion in direct healthcare costs each year[26]. As the population is living longer, there is tremendous interest in discovery of osteoporosis prevention. The discovery of modifiable risk factors in the population, especially in young adult women, may reduce the health care costs and increase quality of life in the postmenopausal years when osteoporosis is most prevalent. The long latent period between peak bone mass accrual and diagnosis of osteoporosis make it is not feasible to conduct randomized controlled exercise intervention trials with osteoporosis as an outcome. Because the physiologic changes that initiate osteoporosis may take place pre-menopausal, even if the symptoms of the disease does not become evident until after menopause, it is important to examine these changes among young women. Therefore, we propose to examine the effects of mechanical loads, estrogens and menstrual cycle characteristics on bone regulation mechanisms observed to be associated with the risk of osteoporosis incidence.

2.1.2 Role of physical activity and hormones for optimizing bone health in premenopausal women

There are several physiologic mechanisms by which exercise may alter bone health. Inducing strains above the MES may cause bone formation through increased mechanotransduction leading to an osteogenic response. Increasing lean mass (a surrogate of the load on the bone), altering of estrogen and other sex hormones, and changing levels of chemicals associated with bone growth (such as IGF-I, GH, and PTH), may all effect bone.

Understanding the importance of the effects of estrogen on bone is becoming increasingly clear. The effects of this hormone on bone turnover, density, structure and strength is significant. Estrogen affects bone both indirectly and directly. It is known that estrogen decreases bone turnover and increases endocortical contraction (packing of the bone on the inner surface, next to the marrow space, of bone), thus maintaining bone mass [27]. This mechanism is complex and involves interaction with many cells and regulation of several mediators. Estrogen may influence bone growth by controlling production of cytokines, growth factors and prostaglandin. Estrogen suppresses levels of cytokines and specific growth factors (tumor necrosis factors), which are potent stimulators of bone resorption and osteoclast formation[27, 28]. Indirectly, estrogen may increase renal calcium retention, stimulate calcitonin, which limits bone turnover[29].

Another vital mediator of bone turnover and formation is the estrogen receptor (ER) found on the surface of osteoblasts [15]. There are two main types of estrogen receptors, ER-alpha (ER-α) and ER-beta (ER-β), that stimulate different responses on the cell surface and appear to compete against each other. ER- α appears to enhance mechanically induced bone formation, while the ER=- receptor suppresses this response [22, 30, 31]. Hence, mechanical loading is necessary for these differentiating effects, thus further research on the relationship between loading and circulating estrogen in premenopausal women needs to be further clarified for defining prevention and treatment of low bone mass and osteoporosis.

The interactive effects of estrogen and mechanical loading on the skeleton are also not well understood in the young adult premenopausal female population. It is known that each of these variables independently has been observed to increase bone density[14, 18, 26, 32-35] .However, the independent and interactive effects of these variables on bone *strength* and *geometric* properties in the young adult female population is not well understood. Animal studies show inhibitory[30, 36, 37 8-10] and stimulatory[22, 38, 39 11] effects of estrogens interaction with mechanical loading. In humans, research suggests that estrogen-replete women may be less sensitive to loading-induced periosteal bone formation than prepubertal girls [40-42] .

Similarly, the mechanical sensitivity of the aging skeleton appears to be affected by exposure to estrogen, as suggested by increased periosteal expansion after menopause[2, 43, 44 14], presumably as a result of estrogen deficiency. Lack of research evaluating the relationship between loading and sex steroid levels in the premenopausal population leaves many unanswered questions.

2.1.3 Prevention of Osteoporosis in Premenopausal Women

Research shows that adolescence is the most critical time for the attainment of peak bone mass (and this increased velocity of bone growth corresponds with the onset of menses) [45-47]. In fact, the amount of bone mineral laid down during the 4-year period of adolescence surrounding peak linear growth velocity is equivalent to the amount of bone mineral most people lose during all of their adult life[46] , and approximately 90% of total BMC is accrued by age 17 [48, 49] . Once women reach peak bone mass, bone loss is usually not recoverable, even with any type of pharmecutical intervention. Thus, one factor in prevention of osteoporosis is increasing peak bone density during the window of growth around puberty. This may be through physical activity (also known as mechanical loading). Mechanical loading may change bone structure and strength, even without significant increases in bone mass. Mechanical loading has a positive effect on bone structure and density in children, especially if physical activity is started 3-5 years prior to menarche[50] , and much of the increased bone mass and geometrical changes as a result of physical activity is preserved over the next 4-5 years[41, 51, 52] . However, it is unknown whether these positive changes are maintained into later life and are thus a factor in reducing osteoporosis risk is unknown.

After menopause, physical activity is able to slow the rate of bone loss found in this population, especially if combined with hormone replacement therapy[9, 12, 53, 54,] and the exercise needs to be of higher-impact or more strenuous[12, 55.] Thus the amount of bone mass and the bone structure at menopause may need to be great enough to maintain strength and density after the initial rapid loss of bone in this population after menopause. It is not clear if physical activity during childhood and/or adulthood can prevent osteoporosis since little is known of the factors that influence bone structure and strength in young women. Some

studies have shown that bone mass can be added as a response to exercise in the female adult and have lasting effects[52, 55, 56] . Unfortunately other studies show that much of the bone mass gained during exercise interventions is lost rapidly once the exercise program ends. Adults who added bone and made positive structural changes during their adolescent growth spurt may maintain those positive effects into adulthood and even into postmenopausal years[52, 57.] Studies also show that lifetime physical activity is a better predictor of bone density and strength after menopause than is physical activity during the adolescent growth spurt [58, 59]. However, others have shown that early physical activity has little effect on postmenopausal bone health[60]. Karlsson and colleagues[60] found little if any reduction in risk of osteoporotic fractures in people who had been active early in life. The conflicting findings in these studies present the need to determine the role of mechanical loading in prevention during the adult years, especially in young women, who are a greater risk for osteoporosis later in life.

2.2 Bone Anatomy and Physiology: What is Bone and Why Do We Care?

2.2.1. Bone Tissue: Composition and Organization

Bone is unique and complex tissue with the primary function of supporting the loads that are placed on it. Accomplishing this function requires having great amounts of strength and resilience, while still being lightweight and metabolically feasible. In addition, bones serve as levers for locomotion, attachment sites for muscles, ligaments and tendons and as a central reservoir for calcium while also providing a site for haematopoiesis (formation of blood cells) and as a protector of organs[61]. During growth, the skeleton must maintain these functions while dramatic changes in size and shape occur.

Within the skeleton, the structure of bone tissue, and of whole bones, is complex and ultimately influences bone's mechanical properties. On a basic level, bone is made up of an inorganic component (70% by weight), organic component (20-25% by weight), and water (5% by weight). The inorganic, or mineral, phase is composed mainly of a specific crystalline hydroxyapatite (calcium and phosphorus). These crystals are found in and around collagen

fibers and give bone rigidity and compressive strength[62]. The organic component is composed mostly by type I collagen, and determines the structure and mechanical and biochemical properties of bone. Along with collagen, the organic compound of bone consists of a small proportion of noncollagenous proteins, which not well understood, are becoming increasingly important in our understanding of the biological and mechanical properties of bone. The combination of these organic compounds and water make up 98% of the *matrix*, in which mineral crystals are deposited[62]. The other two percent of the matrix is made up of the cells of bone: bone-lining cells, osteoblasts, osteocytes and osteoclasts. These specialized cells regulate bone metabolism via responding to chemical, mechanical, electrical and magnetic stumuli[62]

Bone-lining cells cover all surfaces of bone and control the movement of ions into and out of bone. The layer of cells on the outside of the bone is called the periosteum, and the layer of cells on the inside of the bone is called the endosteum. Osteoblasts are derived by bone-lining cells and are responsible for the formation of bone, through initially lying down a collagenous matrix (osteoid), which is later filled in with mineral. Osteoblast function is controlled by endocrine, paracrine and autocrine factors. These cells express receptors for estrogen and 1,25-dihydroxy-vitamin D, which in turn affect response of bone to environmental and nutritional factors. Osteocytes are mature bone cells in the body of the bone, and are buried deep within small bone cavities (osteocytic lacunae). These cells are connected to neighboring osteocytes and with bone-lining cells by long cell processes at gap junctions. The gap junctions allow small molecules through, thus allowing there to be communication between cells. This communication is critical for mechanotransduction (see section 2.3.1). Osteoclasts are the bone-destroying cells and are responsible for removal of old bone, or bone resorption and are usually found in cavities on bone surfaces called resorption pits. They are large multinucleated cells that form by fusion of mononuclear precursors of haemotopoetic origin[3]. Osteoclasts secrete lysosomal enzymes and hydrogen ions that work together to dissolve the bone matrix. During growth, osteoblasts and osteoclasts may function independently to modify the size and shape of bones during bone *modeling*. The osteoclastic resorption is also linked to osteoblastic formation, and this

combination in activity is termed the basic multicellular unit (BMU) which is responsible for bone *remodeling* [9]. I discuss bone modeling and remodeling in further detail in Section 2.2.3.

Bone tissue consists of two types: woven bone and lamellar bone. Woven bone is laid down very quickly with the collagen arranged in a random fashion and is considered immature bone. This type of bone is characteristically found in the fetus (it makes up all the bone in the body at birth), at sites of fracture healing and in response to extreme mechanical loads[63]. Lamellar bone the bone that replaces woven bone, and it is laid much more slowly than woven bone. Collagen is arranged more precisely, with the fibers arranging themselves along lines of force. This allows for bone to be able to withstand forces bettering one orientation than in another.

Woven and lamellar bone are organized into cortical (or compact) and trabecular (spongy or cancellous) compartments. Though cortical and trabecular bone are made of the same material, there are structural and functional differences between them[62]. Cortical bone is made up of dense, calcified tissue, with 80-90% of cortical bone's volume made up of calcium[29]. Cortical bone is arranged in cylinders that align with the long axis of bone and is much less porous (5-10%) than trabecular bone[29]. The inner surface of cortical bone is the endosteum, while the outer surface is the periosteum. Haversian bone is the most complex form of cortical bone. Haversian bone consists of vascular channels circumferentially surrounded by lamellae of bone, known as osteons. The main function of cortical bone is structure and protection[62].

Trabecular bone exists as a three-dimensional lattice structure composed of an inner network of thin calcified trabeculae and is found at the ends of long bones and in the vertebral bodies. Calcium takes up only 15-25% of trabecular bone volume[29]. The lattice organization determines the porosity of trabecular bone (which is 75-95%), provides a vast surface area where metabolic activities such as bone turnover occur and houses bone marrow, which functions in formation of blood cellular components, or haematopoiesis[29].

Taken together, the tissues of bone make up the skeleton at the macroscopic level. The skeleton consists of two parts: the axis and the appendicular skeletons. The axial skeleton includes the vertebrae, the pelvis and the other flat bones such as the skull and scapula. The appendicular skeleton includes all the long bones. Each long bone consists of two wider extremities at the ends of the bone (the epiphyses), an essentially cylindrical shaft in the middle (diaphysis) and the zone between these two sections (metaphyses) where remodeling of bone takes place during growth and development. The epiphyses are separated from the funnel-shaped metaphyses by a layer of cartilage known as the growth plate, which is the site of endochondral ossification. The epiphyses consist mostly of trabecular bone. The outer portion of the diaphysis contains cortical bone, whereas the inside contains bone marrow and is known as the medullary or marrow cavity. The metaphysis is a transitional region of trabecular and cortical bone[62]. The broad shape of bone ends serves to better distribute joint forces and reduce stress (force per unit area) that is transmitted by trabecular bone in the metaphysis to cortical bone in the diaphysis[62].

2.2.2. Whole Bone Strength and Stiffness

The strength of a bone is defined as the load it can bear before breaking, while the stiffness of a bone is defined as the amount of force required to cause deformation[29, 62, 64]. If the bone is not strong enough, its inability to support loads will cause fracture if even it is stiff. On the other hand, if the bone is not stiff enough (floppy), they would not function properly as to serve as a lever arm, and the muscle movements would be futile[62]. The stiffness and strength of bone depends on two factors: the material properties bone material and the geometric or structural properties of the whole bone. The combination of both these properties determines the mechanical competency of the bone.

2.2.2.1 Material Properties of Bone

The *material properties* of bone are the characteristics at the tissue level that contribute to overall bone strength, and are considered intrinsic properties. The material behavior of bone is determined in laboratory experiments done on uniform specimens of bone. The

fundamental concepts to understanding the material properties include stress, strain, mass, and density. Stress is the force applied per unit area and is measured in unites of Newtons per square meter (N/m^2) or Pascals (Pa). Stress on bone can be classified as tensile, compressive or shear which may occur independently or in combination according to specific loading configurations[62]. Strain is the deformation of a material and refers to the relative change in the bone dimension under study, whether that is length, width or angulation. To determine strain, the change in bone dimension is divided by the original bone dimension. The result is a nondimensional measure, and if often expressed as a fraction or percentage, and is reported in microstrains (μe). Strain is greatest at the point of highest loading and dissipates along the length of the bone[62].

The strength and stiffness of the bone can be defined by the stress-strain curve (figure 2-1). In addition to providing quantitative information that is useful for the constitutive relationship, the stress-strain curve can also be used to qualitatively describe and classify the material. Typical regions that can be observed in a stress-strain curve are: elastic region, yield region, plastic region, and failure. Structural component of bone is said to behave elastically if during loading/unloading the deformation is reversible. In other words, when the loads are released the specimen will return to its original, undeformed configuration. The elastic region is defined by the slope of the stress-strain curve, known as Young's Modulus (E), and represents the inherent resistance to loading. This relationship can be represented mathematically as e=S/E (e=strain, S=stress, and E=Young's Modulus). As the loads are increased and the stress in the specimen continues to rise, the material eventually reaches the yield point. Beyond this limit, any additional loading will result in some permanent change to the bone upon unloading. This process, characterized by a near-zero slope to the stress-strain curve, is often referred to as the plastic region. In this so-called plastic region, the deformation will be relatively large for small, almost negligible increases in the stress. If load continues to increase, failure load may be reached, and the structure may fail completely. The area under the curve represents the material toughness; a tougher bone will be more resistant to fracture[65]. The design of long bones is an appropriate combination of stiffness

and toughness which, in the healthy skeleton, allows bones to bear the loads imposed upon them[62]

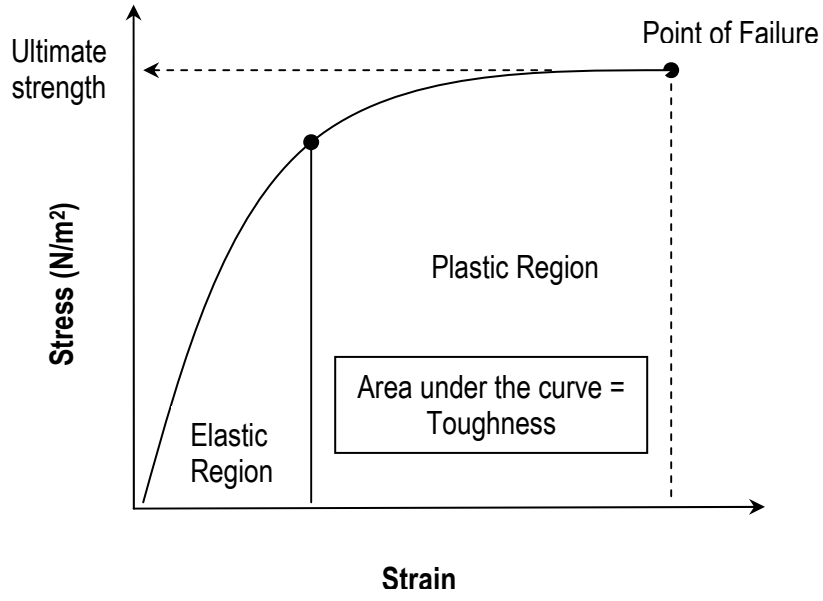


Figure 2-1. A standard stress/strain curve of a bone specimen produced during mechanical testing. This curve can also be used to represent whole bone properties (load/deformation curve). Adapted from Einhorn[66]

Bone's strength and stiffness are also determined by bone mass and bone density. Bone mass explains more than 80% of overall bone strength[67]. Bone stiffness is proportional to bone density cubed and to strength squared. The combination of bone stiffness, strength, mass and density of each bone compartment influences the mechanical properties of cortical and trabecular bone.

2.2.2.2. The Structural (Geometric) Properties of Bone

Structural properties of whole bone include: size, shape, cortical thickness, cross-sectional area (CSA), and trabecular architecture. In the healthy skeleton, these properties adapt to allow bone to be strong enough to resist compression, tension and shear stresses, yet

maintain an a weight that is the most efficient and economic for locomotion. The shapes of bones are varied and intimately related to their functions.

In engineering principles, a hollow cylinder provides the least mass and the greatest strength during bending and torsional loading. This concept applies to the bones of the skeleton[24]. In bending, both the cross-sectional area (CSA) and the distribution of bone mass around the center of the bone (the neutral axis) affect the bone's mechanical behavior and hence bone strength. Both of these concepts are critical for the computation of cross-sectional moment of inertia (CSMI), which is critical to determining whole bone strength, since increases in CSMI decreases bending in response to a give load. In the skeleton, minimal weight and maximal CSMI is achieved when cross-sectional bone area is as far from the neutral axis a possible[24]. CMSI is represented mathematically as $CSMI=(\pi/4) \times (r_1^4 - r_2^4)$, where r_1=outer radius of the bone and r_2=inner radius of the bone. As r_1 increases, the CSMI increases considerably, as CSMI is proportional to the fourth power of the radius. Hence, it is possible to greatly increase bone strength with minimal increases in bone mass or density. Cross-sectional properties (areas, moments of inertia) have been used to address a variety of issues in human skeletal adaptation, including the changes in skeletal structure during growth and development, and the effects of mechanical loading and hormones on the mechanical properties of bone[68-72 10-14].

It hence becomes clear that changes in bone geometry can improve the mechanical competence of whole bone, without much change in the material properties of bone mass and bone density. Therefore, it is important to include estimates of bone geometric properties when determining whole bone strength, since bone strength can result from an increase in bone mass, a positively adaptive change in bone size, shape, architecture, or microarchitecture, or a combination of theses factors.

2.2.3 Bone Growth and Bone Turnover

2.2.3.1 Longitudinal Bone Growth

Many studies on human bone development have focused on changes in bone mass and bone mineral density (BMD). However, describing bone development just in terms of changes in mass or density means looking at bones as if they were simple 2-dimensional objects. Of course, as is well known, bones are complex three-dimensional structures. Taking structural (geometric) aspects into account along with the material aspects allows for a more realistic understanding of bone development[73, 74] .New bone can be added on the outside, which makes bones bigger, or on the inside, which makes bones denser. Bone can be made bigger through the addition of bone mineral to either the length or the width of bones. Specifically, bone growth in length is driven by the growth plate, whereas bone growth in width is the task of the periosteum. The inner bone surface is called the endosteal surface, which can be subdivided into the trabecular, endocortical, and intracortical surfaces [75]. Initially, the cartilage is calcified (primary spongiosum or primary trabeculae) and this allows for the deposition of more calcified material in the form of woven bone (secondary spongiosum or secondary trabeculae). Chondrocytes (cartilage producing cells) within the growth plate are organized according to their stage of maturation, with the most mature cells in the calcifying zone being incorporated into metaphyseal bone[75]. With increasing distance to the growth plate, metaphyseal trabeculae located in the centre of the bone are thinned out and eventually resorbed leaving the diaphysis devoid of trabeculae [76]. In contrast, metaphyseal trabeculae on the periphery serve to transfer loads from the growth plate to the metaphyseal cortex. Eventually the peripheral trabeculae coalesce and become part of the metaphyseal cortex [74]. Growth plate activity varies with age and the contribution of the distal and proximal growth plate to overall longitudinal growth varies between bones [77-79].

Regulation of longitudinal growth is though to occur on three levels: systemic, local and mechanical [76]. At the systemic level growth hormone (GH), insulin-like growth factor-I (IGF-1), thyroid hormones and glucocorticoids regulate longitudinal growth during childhood, whereas sex hormones (estrogen and testosterone) are more influential during puberty[79]. Little is known about mechanical regulation of longitudinal growth; however, mild tension and compression are thought to increase longitudinal growth while severe compression is thought to inhibit growth [76].

After the growth period, bone size changes only slowly [80]. Consequently, bone growth in size is one of the most important determinants of bone strength throughout life. The growth of size and changes in shape are a result of modeling and remodeling, as will be discussed in the following sections.

2.2.3.2 Bone Modeling

During growth, the size and shape of bones is modified through organized bone cell activity referred to as *modeling*, which involves the independent actions of osteoblasts and osteoclasts on bone surfaces [81]. The adjustment of bone shape and size ultimately allows for changes in bone strength. Continuous addition of bone to the periosteal surface by osteoblasts and simultaneous endosteal resorption by osteoclasts contributes to diaphyseal enlargement. The formation and resorption occur on different surfaces, thus surface activation following modeling can be followed either by formation or resorption [82, 83] .In fact, complete unloading may cause resorption not followed by formation [84,85] .

As these processes occur, thought to be regulated by mechanical strain [86], bone modeling causes the surfaces of the bone to move as a means of increasing strength of the bone is areas of highest loads. This motion of surfaces in tissue space is known as *drift*, and may increase or decrease bone curvature according to the specific mechanical needs of the bone [62]. Once skeletal maturity is reached, the modeling rate is greatly reduced. At this time point, another process called *remodeling* begins and is the process of bone maintaining the mechanical integrity of bone through prevention and repair of fatigue damage [62].

2.2.3.3 Bone Remodeling

Remodeling is organized bone cell activity in which resorption and formation are balanced. In this process, old bone is continuously replaced by new tissue. The remodeling process begins at bone surfaces with the appearance of osteoclasts. They attach to the bone tissue matrix and form a ruffled border at the calcified bone surface and create the extracellular bone resorbing compartment. This “sealing” of the osteoclast to the bone creates an isolated

microenvironment. Following the attachment of the cell to the bone surface, the osteoclast synthesizes and secretes lysosomal enzymes which acidifies the microenvironment and dissolves the organic and inorganic matrices of the bone [87]. After this resorptive process stops, osteoblasts appear at the same surface site.

The osteoblasts produce new bone through mineralization of the osteoid (the term for an osteoblast prior to calcification). This process takes approximately 10 days [29]. Some of the osteoblasts are become embedded deep within small bone cavities (the osteocytic lacunae) in the osteoid matrix and differentiate to osteocytes. The remaining osteoblasts continue to synthesize bone until they eventually stop and transform to bone lining cells that completely cover the newly formed bone surface. These lining cells are highly interconnected with the osteocytes in the bone matrix through a network of canaliculi [62].

It has been shown through histological studies that osteoclasts and osteoblasts closely collaborate in the remodeling process, hence when resorption increases, formation increase as well. The pairing of these two mechanisms is called a "Basic Multicellular Unit", or BMU, and can be thought of as a packet of bone being resorbed or rebuilt [9]. The exact nature of this coupling mechanism, however, is not known. The organization of the BMU's in cortical and trabecular bone differs, but these differences are mainly morphological rather than biological. In cortical bone the BMU forms a cylindrical canal of about 2000 μm long and 150-200 μm wide. It gradually burrows through the bone with a speed of 20-40 μm/day. The burrowing of the osteoclasts dig occurs in a circular fashion creating a cutting cone shape through the bone and in the dominant loading direction. They are followed by several thousands of osteoblasts that fill the tunnel (closing cone) to produce a (secondary) osteon of renewed bone [88]. About 2% and 5% of cortical bone is remodeled each year.

The remodeling process in trabecular bone is mainly a surface event. Due to the much larger surface to volume ratio, it is more actively remodeled than cortical bone, with remodeling rates that can be up to 10 times higher [89]. Again osteoclasts come first in the process. They travel across the trabecular surface with a speed of approximately 25 μm/day, digging a

trench rather than a tunnel, with a depth of 40-60 μm. Like in cortical bone they are followed by osteoblast bone formation. Active remodeling sites cover areas of varying sizes from as small as 50×20 μm up to 1000×1000 μm [81]. The resulting structure that is formed is called a trabecular osteon or hemi-osteon [62].

Bone modeling provides a way to the body to shift the balance of minerals in serum, allows the skeleton to adapt to its mechanical environment, and provides a means to repair bone damage (microcracks). Research suggests that to obtain adaptations to load and repair of damage, there must exist both targeted and nontargeted remodeling [90]. However, the signaling mechanisms behind the different types of remodeling are not well understood. It is suggested that the remodeling process provides a means to keep bone strain at an optimal level by adjusting bone structure.

During remodeling, a number of BMUs are in the resorption phase, while others are in the formation phase. Therefore, at sites where remodeling is occurring there is a temporary loss of bone, or undermineralization [91]. The immature skeleton tends to be more undermineralized than the mature skeleton due to the high rate of BMU activation associated with rapid longitudinal growth. This temporary deficit will thus result in a low stiffness and higher strains for a given load. The increased strains may in turn increase fatigue damage and lead to increased activation frequency. The concept of adapting bone to the loads is termed the *mechanostat* [9], which will be discussed in the following section

2.3 Theoretical Framework: Bone's Response to Mechanical Loading

2.3.1. Response of Bone to Mechanical Loading at the cellular level: Mechanotransduction

2.3.1.1. Introduction and Background

Since the days of Galileo in the 17th century, scientists have known that bone responds to physical activity, and later it became obvious that mechanical forces don't only cause a

response in bone, but that these forces are a major influence on the bone modeling and remodeling processes in both cortical and trabecular bone [92]. How bones respond to mechanical loads is a process known as *mechanotransduction*. When a bone is loaded, a vast network of osteocytes and bone-lining cells sense the load (which causes the bone to bend or deform in some way), which in turn signals the appropriate cells to either remove or add bone at the specific sites of loading. Successful mechanotransduction the interaction among four mechanisms: mechanocoupling, biochemical coupling, transmission of the biochemical signal and the effector response [29].

2.3.1.2. Mechanocoupling: The Process of Communication to Bone Cells

Mechanocoupling is the process of communication to the bone cells that the bone has been loaded.[39] This processes involves a physical transduction of a local mechanical force (strain) into a form that bone cells can read and respond to effectively and efficiently. The peak levels of these strains range from about 400 to 2000 μstrain in humans under varied activities (where 1 μstrain equals 1 μm of deformation per meter of length) [62] .Dynamic loading of long bones combines bending and compressive forces creating bone deformation. This deformation creates pressure gradients within bone canaliculae and interstitial spaces, which causes tissue fluid to move. As it flows by osteocytes, it creates shear stress within the membranes of the cells [33, 93], thus we may assume that osteocytes and bone lining cells act as the sensors of local bone strains because they are appropriately located in the bone for this function [94, 95]. As the dynamic loading of the bone increases, cellular stimulation of bone formation increases. However, mechanically induced bone formation occurs only once a certain threshold of strain magnitude and strain rate is reached. More on strain magnitude and rate to follow.

The opposite of loading, unloading or disuse, causes the opposite process to occur. The disuse reduces the deformation of bone, thus decreasing the amount of fluid flow through bone tissue, causing a cellular response for bone resorption. This effect has been supported by studies that simulated weightlessness in which rats were exposed to tail suspension [96]. In these studies, a fluid shift occurred causing increased extracellular fluid pressures and

perfusion in the head. This hypothesis is supported by others [97], suggesting that the decrease in hind limb bone mass during tail suspension studies is caused by a decrease in fluid flow in these regions. The transduction of mechanical forces to signals detected by bone cells is a complicated issue, and what constitutes the signal remains unknown.

2.3.1.3. Biochemical Coupling: Conversion of Mechanical Loads to Biochemical Signals

In biochemical coupling, the initial detection of mechanical forces is converted into a biochemical signal. There are several possible mechanisms involved with this process. One possible pathway is the extracellular matrix-integrin-cytoskeletal axis. Bone cells attached to the collagen matrix by binding integrins, which are also attached to the internal cytoskeleton, and the cytoskeleton connects the extracellular matrix to the cytoplasm and the nucleus [94]. The cytoskeleton maintains tension on the extracellular matrix, and due to this tension, physical stimulus can be rapidly transmitted to the nucleus, possibly altering gene expression[94]. Recruitment and/or differentiation of osteoblasts and osteoclasts are modulated by cellular adhesion to the extracellular matrix [62].

2.3.1.3.1. Transmission of the Biochemical Signal

Osteoblasts are stimulated in two ways once the bone undergoes deformation as a result of mechanical loading. First, active osteoblasts and bone-lining cells can respond directly to the strain[94, 97]. However, only 5% of the bone surface is made up of osteoblast, thus formation would be too slow to be effective. Given that 95% of the bone surface is made up of bone-lining cells and osteocytes, there must exist a form of communication between these cells and other bone cells. This communication occurs through an extensive network of cellular processes connected at gap junctions [29]. However, even with the added effort of all the bone surface cells, they cannot produce substantial amounts of new bone matrix. Therefore, a second pathway of communication of the strain stimulus exists which involves communication of a biochemical signal from nonproliferative, strain sensing cells (osteocytes and bone lining cells) to osteoprogenitor cells and osteoblasts through paracrine factors. These intermediary biochemical compounds are called second messengers [29]. Osteoblasts

are thus also able to communicate directly with osteocytes through the biochemical pathway. These osteocytes than produce anabolic growth factors that are transported to the bone surface for use in bone formation [97].

2.3.1.4. The Effector: Production and Rearrangement of Bone

An effector is required to complete the mechanotransduction process, and this effector is responsible for the production and/or the rearrangement of bone. After a single period of mechanical loading, osteocytes and bone-lining cells release prostacyclin, which is followed closely behind (about 5 minutes) by an increase in glucose-6-phosphate dehydrogenase, and 6-24 hours later by increases in RNA synthesis and IGF-I messages in osteocytes [29, 98]. After 3-5 days, collagen and mineral apposition increases are observed on the bone surface[6]. Bone formation rate was significantly increased in the period between day 5 and 12, largely due to increases in bone-forming surface [29, 97]. These results suggest that each loading bout activates a packet of osteoprogenitor cells that differentiate and start forming osteoid about 4 days after activation. It takes 60 to 72 hours an osteoblast to mature.

2.3.2 Response of whole bone to changes in mechanical loading: The Mechanostat

In terms of mechanical function, the skeleton provides rigid levels for muscles to act against as the work to hold the body upright in the presence of gravitational forces [99], and for locomotion. The ability of bone to adapt to its physical demands has been recognized for over a hundred years ago by Julius Wolff, who was influenced by the work of Wilhelm Roux (1885). Roux theory of the relationship between physical loads and bone structure later was termed the *bone functional adaptation.* Later, Wolff hypothesized that bone optimizes structure so as to withstand functional loading and ensure the metabolic efficiency of locomotion. This adaptation of bone to support the loads placed upon it is the function of the bone cells involved in the processes of modeling and remodeling to ensure proper adaptation of the structural and material properties. For example, an increase in bone strain results in bone formation, which in turn reduces bone strain to its original customary level, but a decrease in bone strain results in bone resorption, which again restores strain to its customary level. This looping of adaptation serves to maintain an equilibrium or customary

strain level when exposed to bone strain. This theory, termed Wolff's Law, later influenced the scientist Harold Frost, who coined the term "mechanostat" to define the regulation of bone according to specific thresholds[9]. The control of the skeleton involves the interaction among feedback loops that are influenced by both mechanical and nonmechanical factors. Also, Frost proposed that there exist more than just one customary strain level, but instead a threshold range that includes a minimum effective strain (MES) for both remodeling (MESr) and modeling (MESm). Once the thresholds are met, bone modeling or remodeling is initiated. This regulatory system provides the mechanism for functional adaptation in the skeleton, so that the mass of load bearing tissue we carry around is not so excessive as to waste energy in growth, maintenance and use [100]. However, the skeleton must be sufficiently strong to provide a safety margin that protects against fracture in response to unlikely but occasional high loading events such as those experienced in falls and impacts [62]. The feedback loop between bone deformation (tissue strain) and bone strength is the essential mechanism of bone regulation. This homeostatic system is continually forced to adapt to external challenges during growth. The process of the mechanostat is shown in Figure 2-2.

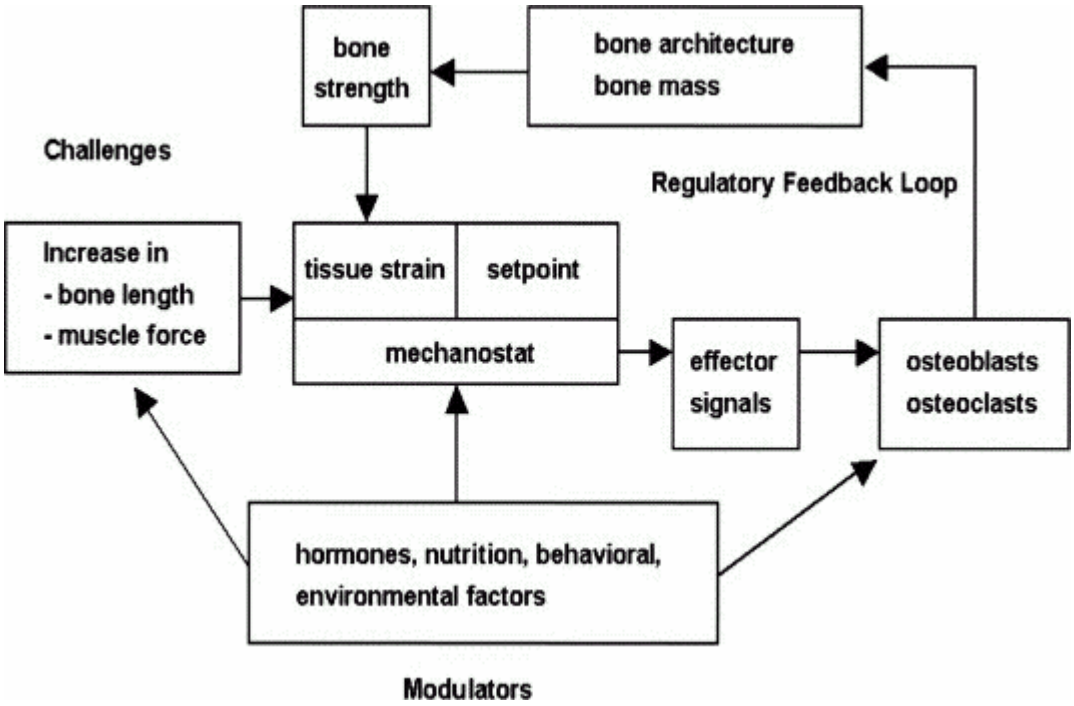


Fig.2-2 A functional model of bone development based on the mechanostat theory. Factors shown below modulate various aspects of the central regulatory system, which will be discussed later. [Adapted from Pediatric Research 2001; 50(3):309-314].

Support for the mechanostat comes from several animal studies [71, 101, 102]. Recently, Frost updated the factors related to the mechnostat[10]. In his update, he verified and updated the many factors involved with the healthy skeletons adaptation to mechanical loads. As known, healthy load-bearing bones (LBBs) and their trabeculae have more strength than is needed to keep the typical peak voluntary mechanical loads (TPVMLs) on them from causing nontraumatic fractures, also known as spontaneous fractures. Also, healthy human's load-bearing bones (LBBs) and their trabeculae are strong enough to keep typical peak voluntary mechanical loads (TPVMLs) from breaking suddenly or breaking from fatigue. This concept is termed mechanical bone competence (MBC), and MBC may be used to define bone quality and functional health[10].

Our skeletal baseline conditions are created *in utero*, and thus our gene expression patterns are established by the time of our birth [10]. Those conditions include our basic bony anatomy and anatomical relationships, our basic neurological and muscular anatomy and physiology, and the biologic mechanism that can increase the strength of LBBs after birth. These mechanisms include modeling, formation drifts, and remodeling.

Along with modeling and remodeling thresholds, repeated bone strains can cause microscopic fatigue damage in bones (microdamage (MDx)) [62], and microdamage has a threshold strain range (the MESp) [62]. Normally, living LBBs can detect and repair the small amounts of MDx caused by strains that stay below the MDx threshold. If strains in and above the MESp range continue without allowing repair, enough MDx can be done that it escapes repair and accumulates. Accumulated MDx causes or helps to cause pathologic fractures, non-traumatic fractures in osteoporosis, and stress fractures in athletes, special forces trainees, and horses [10], among other types of bone fractures and/or deformities that are outside the scope of this questions. Fracture threshold is indicated by the symbol Fx.

The strains on bone are of mechanical nature, with the lever-arm and gravitational effects causing muscles to create the largest loads on the load bearing bones and weight-bearing bones [64]. Thus bone's biologic should adapt its strength more to *muscle strength* (and perhaps to muscle power) than to body weight or other sources of bone loads [103, 104], (muscle strength is measured in Newtons, and muscle power in Newton-meters/sec, Joules/sec, or watts). As we age, we notice a decrease in both muscle strength and power. Hence taking all of the thresholds together, we get a general biomechanical relationship: *MESr* < *E* < *MESm* < *MESp* < *Fx (*where E indicates the typical peak dynamic strains on healthy bones) [105].

Healthy LBB design may rank minimizing fatigue failures above providing great whole-bone strength [106]. This may be at least one function of the (E < MESm < MESp) arrangement in the general biomechanical relation. Physiologic muscle loads on LBBs have the properties of magnitude, rate of increase in magnitude, frequency of loading events, accumulated number

of loading events, and power. Extremely low magnitude strains can efficiently increase bone mass if applied at high frequency.

In summary, whole-bone strength has four chief physical determinants [10]: (1) the bones' structural material including the yield point, stiffness, ultimate strength, and fatigue life. (*2*) the amount of microdamage in a bone; (*3*) the amount and kind of bone tissue in a bone (woven, plexiform and lamellar bone, compact and trabecular bone); and (*4*) the cross and longitudinal shape and size of a bone, and how its bone tissue distributes in space.

2.3.2.1. Strain Characteristics Related to a Bone's Response to Loading:

Physiologic mechanical loads on weight bearing bones have the four distinct characteristics of which bone will respond to: (1) strain magnitude; (2) strain rate; (3) strain distribution; and (4) Strain cycles. Each of these characteristics will be discussed below.

2.3.2.1.1 Strain Magnitude: Changes in Bone Length Under Loading

Strain magnitude is the amount of relative change in bone length under mechanical loading. Animal studies show that bone formation increases with larger strain magnitudes [71]. This response is in direct relationship to the mechanostat, in that to keep bone strain within optimal level, the load must meet the minimal effective strain for bone maintenance. Anything above this strain will increase bone mass and strength while below this strain, a decrease in bone mass and strength. Specifically, any load of <50 – 200μs, where μs=microstrain, remodeling occurs producing a net loss of bone (studies of bedrest [107] and space flight [108] support this theory). Strains in the range of 200-2000ue are in the physiologic loading zone and maintain remodeling at a steady state to maintain bone strength [93, 109]. Loads of 2000-3000μs are considered to be in an overuse zone and modeling is stimulated to form new bone. However, loads over 4000μs causes microdamage that is accumulated can result in fracture. In the microdamage state, modeling begins to lay woven bone down quickly and in an unorganized fashion. Activities of high strain magnitude may have a greater effect on bone than activities associated with a large number of loading cycle.

2.3.2.1.2 Strain Rate: Strain Development and Release Rate

Strain rate is the rate at which strain develops and releases. Strain rate is proportional to strain magnitude, thus peak strain magnitude may be as osteogenic and strain rate. Research suggests that high magnitude strain together with high strain rate is the most effective for a maximal adaptive bone response [110]. This explains the finding by researchers that jumping is more osteogenic than running, since jumping creates higher magnitudes and higher strain rates than running, even if done over a shorter period of time [111]. However, if the strain is of an unusual distribution, the strain rate can be lower and still cause an osteogenic response [112]. Extremely low magnitude strains can efficiently increase bone mass if applied at high frequency. However, while 10-20 large loads and strains per week can increase bone strength after a few weeks [111], and even one loading session might do that [113], it takes many hundreds of thousands of repeated very small strains from low-magnitude vibrations to have that kind of effect, and that can take months[10]. Others support the significant role of strain rate on the osteogenic response; Mosley and Lanyon[110] investigated the effects of strain rate on the adaptive modeling response in the ulna of growing male rats subjected to 2 weeks of axial compressive loading. The loading protocol involved three strain rates (low, moderate, high) at a constant frequency of 2 Hz, and similar peak strain magnitudes across the three groups. The strain rate and frequency used were similar to those recorded by strain gauges (implanted on the ulna) during normal activity. At study completion, the high-strain-rate group demonstrated a 67% greater adaptive modeling response (as measured by change in bone volume) than the low-strain-rate group. It is suggested that strain rate may influence the magnitude of the load induced fluid flow, thus impacting the mechanotransduction process[110].

2.3.2.1.3. Strain Distribution: How Strain is Distributed Across Bone Sections

Strain distribution refers to the way strain is distributed across a section of bone. Results of studies related to the adaptive response of bone in animals lead to the concept of the error strain distribution hypothesis [4]. The basis of this theory is that bone cells maintain the skeleton's structural competence by making architectural adjustments to eliminate or reduce perceived deviations from normal dynamic strain distributions [71]. Hence unusual distributions of strains on the bone are more osteogenic than the repetitious strains of everyday activity

and the more unusual the distribution the greater the osteogenic response. Hence, in humans, activities associated with an unusual strain distribution with a lower strain rate may provide the greatest osteogenic stimulus instead of the strain magnitude.

2.3.2.1.4. Strain Cycles: Number of Load Repetitions at a Given Magnitude

Strain cycles denote the number of load repetitions that change bone dimensions at a given magnitude. A minimum number of loading cycles is required for an osteogenic response, however, there appears to be a threshold above which additional loading cycles produce no additional bone formation. Other researchers support this finding [71, 114]. Also of importance to strain cycles, is the rest between the cycles. In adult rats, partitioning a daily loading protocol into brief sessions separated by recovery periods produced greater gains in bone mass, geometry and strength than one single loading bout [115]. After 16-weeks, rats who received 4 bouts of 90 cycles/bout (90 x 4) with 3 hours of recovery between bouts showed a 70% greater BMC, 37% greater CSA and 46% greater minimum second moment of area at the tibial diaphysis than rats who received one uninterrupted bout (360 x 1). Similarly, Robling et al, found that 14 seconds of rest between load cycles resulted in 66-190% higher relative bone formation rates on the loaded tibia of adult female rats. It is suggested that short-term recovery sessions may enhance the recruitment and/or activation of osteoblasts via fluid-flow mechanisms, while long-term recovery sessions may allow reorganization of the actin cytoskeleton[115]. It is not known whether a similar loading protocol is effective in growing animals. It is thought that without enough rest between cycles, the positive bone response is diminished because the bone becomes quickly saturated. Once the bone is saturated, more loading does not further induce positive bone formation. In a now classic experiment, Rubin and Lanyon used the avian ulnar loading model to demonstrate this cellular saturation. The results of this study showed that 36 cycles/day at physiological strain magnitudes (2000 με) were just as effective for creating an osteogenic response as was 1800 cycles/day at the same strain magnitude[71]. Further more, performing more than 36 cycles did not enhance the bone's response. The growing bone also demonstrates saturation of the bone response to loading. Umemura and colleagues[114] assigned immature female rats to one of five jump-trained groups (5, 10, 20, 40, or 100 drop jumps) or a control group. The jump training began

at a jump height of 25 cm and progressed to 40 cm by the fourth week. After 8 weeks of jump training (5 days/week), the 5-jump group showed significant gains in bending rigidity at the femur and tibia, along with an increase in tibia cortical area. Although there was a trend towards an increased cortical area and rigidity with an increased number of jumps, no significant differences were observed between the 10- and 100-jump groups. These results suggest that short loading bouts were just as effective in initiating a bone response as prolonged loading bouts.

2.3.2.2. Other Factors that Influence Bone's Response to the Mechanostat.

It has been established that nonmechanical agents affect osteoblasts, osteoclasts, and/or their precursor cells by increasing or decreasing (re)modeling with or without mechanical loading. These factors include genes, hormones, calcium, vitamins C and D, some drugs, and some cytokines [18, 19, 28, 33, 53, 113, 116, 116-122].

It has been hypothesized that some of these non-mechanical agents directly affect the mechanostat through an increase or decrease the sensitivity or set-point of the bones response to mechanical loading[13-20]. That is, through a mechanism not well understood, certain nonmechanical agents somehow allow a lower amount of strain to stimulate bone formation than what we would expect without administration of the agent, or vice versa (raising the level of strain needed to stimulate formation). Much research has shown the effect on the skeleton without a mechanical intervention[29], but of particular interest to us is the combination effect of these agents and mechanical loading. It is not known if there is an additive effect, a synergetic effect or negative or no significant effect.

Anabolic agents have been postulated to influence loading related bone formation in a permissive manner and modulate the responsiveness of bone tissue to mechanical loading by lowering the modeling threshold and raising the remodeling set points. Much of the research has been done in animals. Prostaglandin E2 (PGE2) effects repeatedly revealed a greater additive osteogenic response in the more heavily loaded parts of the skeleton than compared to similar loading in rats that were not administer PGE2 in rats [123, 124], adult dogs

[125], and humans [120, 126, 127]. Parathyroid hormone (PTH) also shows a positive synergetic effect of combining PTH with mechanical loading in rats [128] Interestingly, when the rats were given PTH and were not subjected to loading, there was no significant effect of the agent alone [128].

The combination of growth hormone (GH) with mechanical loading also showed a positive response. This can be achieved because GH levels could alter the cellular microenvironment by direct action on osteoblasts or indirectly through GH stimulation of IGF-1 production. Yeh and colleagues[129, 92] reported that GH had an additive effect with the treadmill exercise on tibia cortical bone formation resulting in a synergistic interaction on both the endosteal and periosteal surfaces of the cortical surface, this was supported by another study of treadmill exercise with GH administration. Oxlund and colleagues[130] also showed that GH and treadmill exercise markedly enhanced cortical bone formation and strength in older rats. Others who used voluntary exercise with GH administration support this finding. They also found an additive effect with the main effect was increased periosteal new bone formation[131]. Unfortunately, there lacks data on the direct effect of IGF-I combined with mechanical loading on the skeleton, but close relationship between GH and IGF-I may be indicative of a similar effect between IGF-I and loading as there is between GH and loading.

Of particular interest of both researchers and clinicians is the relationship between sex steroids and mechanical loading. Some research suggests that the effects of estrogen and loading are additive [34, 132, 133], while other suggests a negative effect [37, 134]. It has been established that estrogen maintains bone mass through depression of bone resorption[29,20]. Whether estrogen facilitates the bone anabolic response to increased mechanical loading is unclear. Mechanical loading, when combined with estrogen, results n a greater osteogenic response than does either condition separately. However, whether the osteogenic response of increased bone mass actually increases bone strength significantly is the question that needs to be addressed. As mentioned previously, and increase in bone mass significantly effects bone strength when the bone mineral is deposited on the periosteal surface. Unfortunately, estrogen is known to create bone formation and packing on the endocortical

surface, thus making increases in bone mass questionably as to whether it impacts bone strength[29].

Studies have found that estrogen reduces periosteal bone formation in rapidly growing rats [37, 134] followed by several reports that estrogen dampens periosteal modeling response to loading in older female rat skeletons [134] . It is common knowledge that ovariectomy leads to increased bone turnover in all envelopes, which could suggest estrogen decreases bone turnover. This may suggest that estrogen administration suppressed and ovariectomy enhanced mechanically induced ontogenesis. There is no direct evidence that estrogen enhances the responses of bone cells to loading. The available evidence suggests estrogen has no or a depressive effect on the adaptive response to loading by individual cells. This led researchers hypothesize that it is not estrogen that is necessary for a competent adaptive response to load bearing but the estrogen receptor [30, 34]. More research is needed to determine the exact mechanism behind estrogen's regulation of bone (re)modeling, but the area of estrogen receptors looks promising.

The independent effect of progesterone, androgens or testosterone when combined with mechanical loading is not well understood. In a study of rowers, those with the least amount of progesterone had the least amount of osteogenic response to the physical activity, but more than that of controls [135]. Prior and colleagues[124] research on progesterone and bone indicates that progesterone appears to act directly on bone remodeling and may play a role in the coupling of bone resorption with bone formation. This observation raises the question about the role of progestins on bone mass preservation. Along with progesterone, the role of androgens is also of interest. Androgens are fount extensively in both females and males. Androgen receptors are found in all three types of bone cells: osteoblasts, osteoclasts and osteocytes, but are mainly expressed in osteoblasts. Specific binding receptors for testosterone have been identified [34]. Androgens stimulate osteoblast proliferation, enhance osteoblast differentiation and the synthesis of extracellular matrix proteins, and stimulate mineralization[102]. The role of testosterone seems to differ between the sexes when combined with loading. In males the effects of estrogen and testosterone add to, but do not enhance,

the osteogenic responses to loading, but in females the effect of testosterone was no greater than just loading alone [38, 136]. However, there is limited amount of quality data looking at the relationships between testosterone and loading.

2.4 Measurement of Bone Mass and Strength

The amount of bone within a cross-sectional area affects bone strength, with more bone equalling a stronger bone. The amount of bone, reported as bone mineral density (BMD) is most commonly measured by dual-ray x-ray absorptiometry (DXA)[24]. However, the two-dimensional nature of DXA actually provides us with only an estimate of the areal BMD (aBMD), which represents the average mass thickness (g/cm2) over a given length of bone. Areal BMD contains no information about how the bone material is distributed (bone geometry), which would require a volumetric measure (i.e. g/cm3). Recent advances in technology, including peripheral quantitative-computed tomography (pQCT), allow for more accurate measures of the geometrical properties of bone. The pQCT is able to distinguish bone compartments and to measure density volumetrically (g/cm3) rather than over projected areas as in DXA, resulting in more precise estimates of volumetric density (vBMD) and bone geometry [137] , and measuring bone geometry is the most accurate way to determine bone strength. Therefore, in order to understand the changes in bone strength that occur during aging, it is essential that the geometrical properties of the skeleton be assessed. This thesis will involve the use of DXA to determine body composition (lean and fat mass) and the pQCT to assess bone density, geometry and strength. I discuss the strengths and limitations of these technologies in this section.

2.4.1 Dual Energy X-ray Absorptiometry (DXA)

Currently, dual energy x-ray absorptiometry (DXA) is the most commonly used modality to assess bone mineral status of the skeleton and is the most relied on tool to provide quantitative and meaningful measure of bone mineral density (BMD) in both clinical practice and research [138]. DXA is a relatively inexpensive, noninvasive technology that requires a short scan time and is associated with low radiation exposure [138]. The outcome variable of bone mineral content (BMC) in grams represents the attenuation values of photons that pass

from an X-ray tube (source) through the region of interest. For each region, the projected, 2-dimensional area of bone (bone area, cm2) analyzed is used to calculate the areal bone mineral density (aBMD, g/cm2). Research has shown that DXA-derived aBMD is a reasonable predictor of bone strength, and ultimately fracture risk [139, 142]. In a clinical setting, the precision error is 1-2% for repeated measures[139].

However, in recent years, DXA has been shown to several limitations. Over the range of soft tissue anthropometrics typical of adult patients, the overall percentage inaccuracies in DXA-measured BMD are can be quite sizable and are shown to vary considerably for different bone structures[143]. For example, at the typical lumbar vertebral bone site, BMD inaccuracies are found to be as large as 25% for normal patients, over 35% for osteopenic patients, and close to 50% for osteoporotic patients. In bone structures made up of mostly trabecular bone (e.g., distal radius and distal tibia; both which are bone segments measured for my thesis), it is shown that BMD percentage inaccuracies approach 20% for normal patients, 25% osteopenic patients, and almost 35% for osteoporotic patients[143]. In bone structures made up of mostly cortical bone (e.g., mid-shaft radius and tibia; both measured for my thesis), the BMD % inaccuracies are comparatively small, being less than approximately 2% [143]. Hence, the range in accurate readings depends not only on bone site, but also on current bone health of the patient.

Also, the planar nature of the measurement is a considerable limitation[139]. The DXA-derived "BMD" value does not correctly represent the areal density of bone mineral material, as it is contaminated by sizable, unavoidable, inextricable, independent soft tissue contributions[138]. Of particular relevance to longitudinal studies, DXA is unable to account for changes in bone size and geometry that occur during growth[144]. As a result, BMC and aBMD measures in a woman with short stature and smaller bones will likely be underestimated while the opposite is true for a woman with a taller stature and bigger bones. To correct for the third dimension, a mathematical equation can be applied to the DXA outcomes to generate bone mineral apparent density (BMAD), or the amount of BMC per total bone volume[145]. Underlying this correction is the assumption that bone cross-sectional shape is geometrically similar between

subjects, and that bone thickness scales linearly with the measured projectional area[140, 145]. Although this assumption may hold true for skeletal sites that are considered cylindrical (femoral neck), it is likely not appropriate for more complex geometries such as those of the lumbar vertebrae[140]. While studies using DXA make an important contribution to the field, it is important to recognize the inability of this technique to independently measure structural strength and material bone properties.

2.4.2 Peripheral Quantitative-Computed-Tomorgraphy (pQCT)

Given the limitations of DXA, 3-dimensional imaging modalities such as peripheral quantitative computed tomography (pQCT) are being employed more frequently in bone research. Peripheral QCT was developed specifically as an extension of the larger QCT systems, which are able to measure (volumetric) bone density in the axial and appendicular skeleton. Although both instruments are unique in their ability to separate cortical and trabecular bone, pQCT has several advantages over QCT including higher resolution, higher precision, lower radiation and lower cost [146, 147]. Also, unlike DXA, in which bone density measurements are affected by skeletal size and changes in density may be obscured by changes in skeletal size, the pQCT's measurement of volumetric BMD is independent of skeletal size. The UMN Laboratory of Musculoskeletal Health owns a pQCT device, specifically one of the XCT 3000 machines (Stratec Medizintechnik GmbH, Pforzheim, Germany), and thus I focus my discussion of pQCT on this model.

Similar to DXA, pQCT measures the attenuation of radiation as it passes from the source to the detector through the object of interest. However, unlike DXA, pQCT scans a single tomographic slice and is used to measures volumetric bone mineral density (BMD) (mg/cm3) and cross sectional bone dimensions at peripheral skeletal sites: the radius and tibia. The tissue level density reflects both the degree of mineralization of organic bone matrix and the porosity of the tissue at the cortical level and is a measure of bone mineralization as well as the thickness and number of the trabeculae at the trabecular level [148] (figure 2-3.). This has important implications for interpretation of pQCT outcomes. Along with measurements of cortical and trabecular density, the pQCT also can measure bone area, cortical area, cortical

thickness, periosteal and endosteal circumference, muscle cross-sectional area and biomechanical strain strength indices.

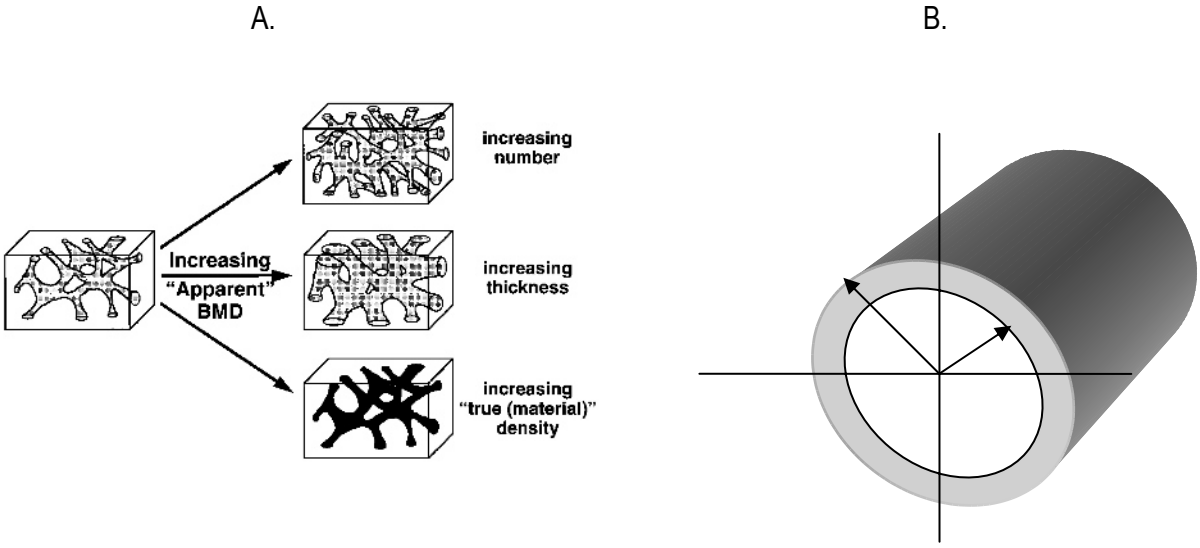
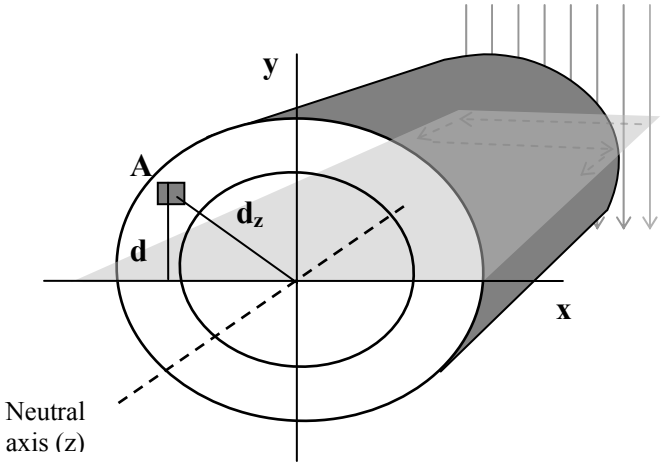


Figure 2-3. A. Schematic showing that changes in vBMD can be due to increases in trabecular number, thickness and/or the actual density of the material (i.e. mineralization). From Seeman, JCEM 1998. B. Three dimensional image of bone allows for measurement of geometric properties and separation of trabecular and cortical bone.

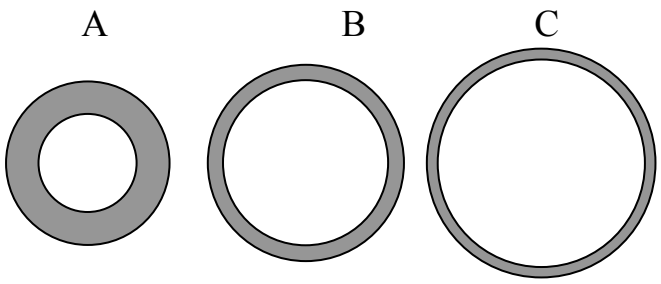


pQCT:

$CSMI_x = \sum (A_i \times d_x^2)$

$CSMI_p = \sum (A_i \times d_z^2)$

Figure 2-4. Schematic of a long bone and its biomechanical properties measured by DXA and pQCT. Whereas DXA calculates bone mineral content (BMC) from planar x-ray attenuation data, pQCT generates a three-dimensional cross-section from which geometric and material properties of the bone are obtained. For example, the bending and torsional cross-sectional moments of inertia ($CSMI_x$, $CSMI_p$) are obtained as the integral sum of the products of the area of each pixel (A_i) and the squared distance (d_x, d_y, d_z) to the corresponding bending (x, y) or torsion (z) axis. Adapted from Kontulainen [149] and Ferretti et al.[150].



	A	B	C
Areal BMD (DXA)	1.00	0.53	0.36
Volumetric BMD	1.00	0.46	0.27
Section Modulus (Z)	1.00	1.00	1.00
Bone CSA (or BMC)	1.00	0.66	0.53

Figure 2-5. Schematic representation of 3 bone cross-sections with expanding periosteal diameter (from A-C) and constant section modulus. The areal BMD (by DXA) or volumetric BMD (by QCT) is reduced (A-C) despite the same bone bending strength (section modulus). This is because the contribution of the bone surface to the section modulus varies exponentially with distance from the center of mass of the cross-section; as diameter is increased, less material is needed for the same bending stiffness.

Unlike DXA, pQCT offers the operator a number of choices related to scan acquisition parameters. These include the resolution, scan time, reference line placement and scan site.

Although these parameters influence outcomes of interest, it is uncommon for researchers to report their acquisition protocol(s) and there are currently no standardized protocols for pediatric pQCT studies. This poses significant challenges when comparing results across pQCT studies.

As discussed, pQCT offers several advantages over conventional DXA measures of BMC in the assessment of adult bone. In addition to bone areas, densities and estimated strength, pQCT can also estimate muscle cross-sectional area (MCSA). With the exception of a lack of standardized acquisition and analysis protocols there are few limitations of pQCT. One concern for longitudinal studies is the long-term precision of pQCT measurements. It is not possible to reproduce the same exact location over time. However, the same relative location along the bone length can be determined using a fixed anatomical reference line. However, the same relative location along the bone length can be determined using a fixed anatomical reference line. Also, there are currently no standardized protocols for adult pQCT studies. This poses significant challenges when comparing results across pQCT studies.

2.5. Mechanical Loading and Bone Strength

"Every change in the form and function of bone or of their function alone is followed by certain definite changes in their internal architecture, and equally definite alteration in their external conformation, in accordance with mathematical laws" (Wolff 1892)

Although Wolff propsed the form follows function relationship of bone, known as Wolff's Law, over a century ago, scientists still believed for a long time that bone architecture, health and disease depended mainly on nonmechanical factors. These nonmechanical factors influenced bone's effector cells (obsteoblasts and osteoclasts), and that these cells functioned independently of each other. However, today evidence strongly suggests that mechanical loading is the primary factor in causing changes in bone growth and development [103, 151]. Though it is important to point out that the effects of loading on bone remodeling follows a U-shaped curve—remodeling is increased in disuse (insufficient loading) or overuse

(overloading causing damage), thus reaffirming the fact that bone is constantly changing in response to the loads placed upon it (or lack of loads).

The greatest changes in bone architecture and strength occur during puberty, which is a time of rapid growth. Adults who became active after a sedentary childhood tend to slow the rate of endosteal resorption, but are not able to add considerable amounts of new periosteal bone[152-155]. These different responses to similar stimuli are that mechanical loads prior to skeletal maturity will result in greater periosteal expansion of a long bone's shaft and less endosteal addition; while mechanical loads during adulthood have little effect on the periosteal growth of long bones diaphyses, but result in greater cross-sectional areas from increased endosteal formation [61] . Biomechanically, the adult response is less efficient, because the contribution of a given unit of area to an endosteal surface increases bone strength in proportion to the square of its distance to the neutral axis[24]. Thus a small addition to the inner surface of the bone will not nearly increase bone strength as significantly as that same amount of bone laid on the outer surface of the bone. Nonetheless, even after puberty, bone does respond to loading and the skeleton continues to optimize its architecture by subtle adaptations to these mechanical loads.

2.5.1 Bone adaptation to mechanical loading: Premenopausal Women

As previously discussed (section 2.3.1), the conversion of mechanical forces into a cellular response is known as mechanotransduction. Age is speculated to modulate the skeletal sensitivity to mechanical loading, as physical activity interventions have shown to significantly increase bone gains in the young (growing) skeleton. Bone tissue has the capacity to respond to exercise not only during skeletal growth but also in adulthood. After puberty, the effects of physical activity in women have been controversial, with minimal (if any) increases in BMD with physical activity[50, 156-159]. Typical increases in BMD among adult exercisers are relatively small with only a few percentage increase in BMD[160, 161]. Longitudinal studies of premenopausal women in a bone-loading intervention show an increase of 1-3% at the loaded sites compared with controls. Other studies show that instead of an increase in the

exercise group, there is reduction of loss in the exercise group compared to sedentary controls[162]. To date, some of the best evidence of augmentation or preservation of bone mass in premenopausal women is provided by several studies done by Friedlander and colleagues at the UCLA[8, 54, 163-165]. A recent meta-analysis of the effect of exercise training interventions on changes in bone mass indicated that randomized controlled trials show consistently that the exercise prevented or reversed about 1% of bone loss per year at both the lumbar spine and femoral neck in premenopausal women[159]. Hence, it appears that instead of large changes in BMD seen in pubertal children put through an exercise intervention, evidence suggests that young adults tend to mainly preserve rather than increase BMD with weight-bearing physical activity.

The range of differences in outcomes seen between exercise intervention study may be a result of the nature of the intervention, since the study duration, intervention type, subject's ages, and intensity of exercise. Those that found increases in bone mass included intense weight-bearing exercises such as jumping[8, 54, 163, 166]. Jumping can created ground reaction forces up to six times body weight. Other studies used high-intensity weight-training[164]. In studies using low-impact exercise, no change or a decrease in BMD was seen[162, 167]. Sports where impact loading is high, such as athletes who play volleyball, basketball or are gymnasts, significant increase in BMD was seen[168-170]. Volleyball and basketball players had 11 and 7% higher lumbar spine bone density than nonathletes and 21 and 29% higher calcaneal density[168]. College gymnasts had lumbar spine bone density 8% higher and femoral neck density 15% higher compared to sedentary controls[169]. In one study 56 were randomized women to either a high- or low- impact exercise group in which only one side of the body was loaded. The low impact group performed 3 x 20 repetitions of strength training exercises at a low intensity weight while the high impact group 3 x 8 repetitions at a high intensity weight. After 12 months, the high impact group had increases of 1-3% at the trochanter and radial sites compared to controls, while the low –impact group showed no changes at any site[171]. These results suggest that high-impact loading can lead to greater increases in bone density during adulthood than any other form of exercise, though more research is needed to determine whether this is true. In regards to the low-impact

interventions, they may not provide enough load on the bone to pass the remodeling threshold required to promote osteogenesis, and even when studies were carried out for several years, bone loss was still detected, suggesting duration of study may not compensate of the lower intensity. However, the length of an exercise intervention study needed to promote bone gain is not known. Research has suggested anywhere from 6 months to 18 months. Given the theory of the bone modeling transient and what is known of bone physiology, it appears that in premenopausal women, an intervention should last a minimum of six to nine months[29].

Of course, as we have read, BMD may not be the best predictor of bone health and bone strength. The above mentioned studies lack the measurement of bone geometry from pQCT or other 3-D measuring tools. Data on BMD are less useful than measures of cross-sectional geometry, and often do not provide information about what surfaces on a bone have grown or been resorbed in response to changed mechanical loading [61]. Thus, little is known about the surface-specific and geometric-specific effects of mechanical loading on the skeleton during adulthood. Women may have changes in the architecture of their bones even without measurable changes in BMD. The small amount of cross-sectional studies of bone geometry provide considerable evidence for important age-related effects on modeling responses to mechanical loading. A consistent finding is that long bone diaphyses grow thicker and stronger as bone length and body mass increases[118, 172]. In fact, body mass emerges as the strongest single predictor of femoral cross-sectional geometry, explaining 82% of the variance in femoral geometry[173]. In general, the most significant change during the premenopausal years with physical activity is an increase in bone size. Bigger bone size enhances the mechanical competence of bone since a larger cross-sectional area can bear larger compressive loads and cope more efficiently with bending loadings[174]. Weight-bearing physical activity causes bending and torsion loading, thus the bone size aids in resisting these types and thus possibly decrease risk of fracture[175]. Thus, it may be that the osteogenic responsiveness to physical activity may differ qualitatively in the premenopausal population instead of quantitatively. The small amount of bone laid down as a response to loading may not be quantitatively significant, but as discussed earlier, where the bone is laid

can significantly alter the geometrical shape of the bone and thus alter the strength of the bone.

A recent study demonstrated an annual decline of about 0.4% in bone mass among sedentary premenopausal women, but only a slight decline in axial strength and no change in section modulus (bone bending strength). However, exercise interventions have shown increased bone mass and bending strength in young adulthood[54, 56, 166, 176]. The enlargement of bone size and the decline in volumetric bone density with age are well-known phenomena[177-180]. The increase in bone size seems to be a partial compensation against age-related bone loss and helps to maintain the mechanical strength of the skeleton. Though the beneficial, although relatively small, effects of load-bearing exercise on the skeleton during premenopause have been shown in many DXA studies[181, 182], the specific effects of PA on bone volumetric (trabecular or cortical) density and size or bone structure as assessed by pQCT are very scarce[55, 180, 183, 184]. A study by Kontulainen et al.[185], that used pQCT to assess cross-sectional properties in the humeri of female tennis and squash players found a substantial increase in bone strength in the tennis players over controls, with markedly greater difference between the players and the controls of girls who had started training at or before menarche. Similar findings were found by Bass et al. in a study of pre- and post-pubertal tennis players[40].

Of interest is the finding that weight bearing PA seemed to be associated negatively with the bone mass of the radius in women. In fact, PA seemed to increase bone loss in the nonloaded radius[54]. Also, in the cross-sectional study of athletes, in which training did not intensively load the upper limbs, a lower radial bone density was evident than compared with sedentary referents[186]. These observations are suggestive of a “steal phenomenon” or redistribution of bone mineral from non-loaded sites to loaded sites.

In the loaded sites, specifically the tibia (the site measured in my study), the trabecular density of the distal area is associated with PA in premenopausal women[187]. However, it should be remembered that the detailed trabecular structure underlying the pQCT-derived

trabecular density could be the result of increased trabecular thickness, trabecular number, connectivity, anisotropy, plate, or rod type of trabecular architecture, and thus its influence on the actual mechanical competence of the given bone remains unclear.

Nonetheless, the differences in bone geometry caused by PA increases estimated bone strength and these changes in bone geometry and strength are site specific. These positive effects of PA on bone are largely the result of changes in bone size and structure rather than changes in volumetric bone mineral density[13, 54, 55, 187].

2.5.2 Bone adaptation to mechanical stimuli: Animal models

Though data is very limited for the effects of loading on adult premenopausal bone geometry and strength, animal models clearly show positive geometrical changes with loading. Similar to those studies in humans, the results from studies of animals also support the notion of maturity-dependent preferential change in cortical surfaces with mechanical loading[188, 189]. Younger animals show greater periosteal expansion while older animals show greater endocortical contraction. Recent studies in adult rats show the positive geometrical changes of bone in response to loading. Robling and colleagues[190] cyclic mechanical loads along the ulna of adult rats three times per week for 16 weeks. Under these bone bending loads, there was an improvement in bone structure as evidenced by a 69% increase in second moment of area, a 64% greater bone strength than controls, yet the improvement in bone mineral content (BMC) was only a modest 7%. Therefore, in this animal model, loading induced dramatic improvements in bone biomechanical properties, even with small changes in BMC. The structural efficiency of the ulna was improved by bone formation, specifically in highly stressed areas where it was most needed. The noted change in bone structure without changes in bone mineral content suggests that not only does bone quantity increase with mechanical loading of bone but also bone quality. This theory has been explored by others with similar findings[184, 191, 192]. Thus, geometric structure, internal architecture, and organization of the collagen fibers likely adapt to mechanical loading in such a way as to increase bending strength and resistance to fracture. However, research does show

significant changes in BMD, even though it may not be as significant as changes in structure and internal reorganization[193].

Studies of bone adaptation to loading in rats have made use of a wide variety of exercise protocols in order to compare the effects of different modes, intensities and frequencies of exercise. Jump training in rats, even of just 5-10 jumps per day, resulted in a marked osteogenic response and enlarged the cross-sectional area of the tibial diaphysis via increased periosteal expansion[114, 194-196]. The jump program used in the study by Umemura and colleagues[194] (10 jumps/day for 8 weeks) increased tibial bone mass and strength, as well as the cortical area, periosteal perimeter, and moment of inertia at the midshaft of the tibia, but did not increase the endosteal perimeter at the midshaft. A study of adult humans found that a loading of 300 cycles lasting 5 min resulted in the transformation of bone lining cells into active osteoblasts within 5 days of the load stimulus[197]. In a study of adult female rats, compression of the tibia has created a periosteal bone formation response, and even after 2 hours of the loading session, tissue from the tibia showed increased bone cell metabolism and proliferation in trained female rats that had been sham-operated or ovariectomized[198, 199].

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In other animal research, studies have measured the response of bone exposed to precisely quantified loads at distinct skeletal sites. For example, Rubin and Lanyon applied dynamic loading to pins inserted in the ulnae of turkey [200]. They found that bone growth was directly proportional to the applied load, and that bone growth was stimulated after reaching a threshold. This was also supported by Turner and colleagues, who used a four-point bending apparatus to apply bending forces to the lower right tibia of 9-month-old female rats[201]. They observed a loading threshold for activation of bone formation at about 1050 μstrain. After this threshold was surpassed, there was a linear increase in formation on the endocortical surface. Both of these in vivo models demonstrate a threshold response and a dose-response relationship between loading and bone formation.

Animal studies have also allowed researchers to study changes in trabecular bone and collagen alignment, which may significantly affect bone strength and structure without changing bone mass. The interest in trabecular structures stems from Wolff's law, based on the observation that trabeculae roughly align with the directions of maximum stresses. Trabecular alignment results in a material property called anisotropy, which means that the strength and stiffness of the bone varies with direction. Trabecular bone is stiffer and stronger in the direction of trabecular alignment. By becoming anisotropic, trabecular bone greatly increases its load-carrying capacity without increasing mass, thus improving structural efficiency[202]. Along with trabecular alignment changes, mechanical stresses may also improve bone strength by influencing collagen alignment. In an experiment where stresses were altered in the forelimbs of adult dogs for a period of a year, collagen orientation within the bone was changed. At the medial cortex, tensile stresses were increased substantially, which was associated with an increase in collagen alignment[203]. These results suggest that local mechanical stresses affect the collagen construction during bone remodeling.

2.5.3. Measuring mechanical "loads" on bone

The definition of "load" is not universally defined in the literature in relation to skeletal loading. As discussed in previous sections, stresses or strains are often used as measures of the load used on bone. The stresses or strains in a particular structure (in our case, bones) are compared with the known failure limits for the material. When the internal loads exceed a certain set point, the geometry of the structure must adapt or the external forces reduced. The direct measuring of these "loads" is difficult in humans, given the invasiveness and sophisticated instrumentation and recording techniques required. Even indirect measurements are limited in both the number of subjects tested and the validity of the test. However, the most widely used measures of load in human subjects include muscle mass, total body mass and lean mass and level of weight-bearing physical activity[105]

The largest forces on the skeleton are due to muscle contraction, as virtually all muscles work against unfavorable lever arms and therefore have to amplify any external or internal load that they have to move around. However, it is not necessarily the size of the muscle, but

rather the force produced by the muscle, that drives bone development[151]. A close correlation between muscle size – a surrogate measure of muscle force – and bone mass and strength is therefore expected[19, 204]. However, data are sparse on the sex-specific development of this muscle–bone relation in weight-bearing bones. Since direct measurements of muscle force can only be determined invasively with force transducers, indirect methods such as dynamometry, EMG and muscle-cross sectional area (MCSA), which can be measured by pQCT, are used to estimate muscle force in adults[205-207].

To estimate muscle force, the bending moment that a muscle exerts may be estimated by the product of MCSA and the length of the lever arm (i.e., tibial length)[205]. The relationship between MCSA and muscle force is based on the well known association between the physiological cross-sectional area (PCSA) of muscle, which is defined as the total area of fiber, and the force potential produced by the muscle[208]. Physiological CSA is calculated as the ratio of muscle volume to muscle fiber length, while also assuming a constant area along the entire muscle length. In muscles in which fibers insert into the tendon (e.g. soleus), force per anatomical cross-sectional area depends on the angle of the muscle fibers to the line of action of the muscle (pennation angle). However, in parallel-fibred muscles (e.g., biceps brachii), maximum force increases linearly with PCSA[208].

Studies on adult females support the relationships among pQCT-derived MCSA and bone strength and geometry, though to date, no longitudinal studies exist looking at changes in MCSA and pQCT measures. In a cross-sectional study based on images from the lower leg as assessed by peripheral quantitative computer tomography (pQCT), MSCA and bone measures were done in 39 female and 38 male control subjects and 15 female professional volleyball players, all between 18 and 30 years of age. From these measurements, muscle-bone strength indices (MBSIs) were developed for compression (CI = 100 · bone area/muscle area) and bending (BI = 100 · bone area moment of resistance/muscle area/tibia length). Significant correlations between muscle cross-sectional area and bone were found at all section levels investigated[205].

Another measurement often used as a surrogate for *load* on bone is total lean mass, as measured by DXA. Of the studies looking at the effects of specific tissues on bone, the majority have compared tissue measurements to DXA measurements of BMD. Nonetheless, lean mass has proven to be the most robust predictor of BMD in adults[209-211]. In a cross-sectional study of young women, each kilogram of lean mass was associated with about a 1% greater proximal femoral BMD[211]. It has been suggested that total lean mass may be the most powerful soft tissue determinant of BMD in premenopausal women[186]. Limited studies on total lean mass and pQCT derived measures in adults show significant positive correlations between total lean mass and bone strength, specifically in the radius[212], and in children[213, 214]. However, to my knowledge, these relationships have not been widely studied in the young adult female population.

Along with imaging techniques to measure actual volume of muscle mass, gathering information of current and past physical activity participation of participants can also provide a measurement of the type, frequency and intensity of muscle contractions on bone, all which are significant variables in causing bone adaptation. As reviewed in the previous section, weight-bearing physical activity is significantly correlated with bone measurements of strength and density.

While to date, the measurement techniques used to estimate muscle force are rough and as just stated, estimates, they do provide us with evidence of the significance between muscle and bone. Of course, attempts to model the contribution of a particular muscle to a given movement or specific bone torque will need to consider the percentage and distribution of each fiber type, the functional differences between fiber types, and the level of daily activity generally experienced by the muscle or muscle group.

2.6 Mediating Factors that Influence Bone in Premenopausal Women

2.6.1 Sex Hormones: Estrogen and Progesterone

Sex hormones play an important role in skeletal homeostasis [215], particularly the predominant hormones estrogen and progesterone. Though it is known that these sex hormones play a significant role in bone metabolism and growth, the precise mechanism is still not clearly

understood [15]. Nonetheless, the importance of understanding the role of estrogen and progesterone on bone is becoming increasingly clear, as these hormones significantly affect bone turnover, density, structure and strength.

2.6.1.1 Mechanisms of Estrogen Effects on Bone

Estrogen affects bone both directly and indirectly[29]. It is known that estrogen decreases bone turnover and increases endocortical contraction (packing of the bone on the inner surface, next to the marrow space, of bone), thus conserving bone mass[15]. Estrogen also increases cancellous (trabecular) bone formation and affects the endocortical surface differently from the periosteal surface[17, 215], and affects the metaphysis (growth plate) differently from the epiphysis (ends of long bones)[62]. Estrogen also reduces osteoclastogenesis, which is creation of the bone resorption cells, by modulating the production of cytokines and transforming growth factor from bone marrow and bone cells[16]. Currently, however, the primary skeletal effect of estrogen at tissue level is suppression of bone turnover and maintaining balanced rates of bone formation and bone resorption[15].

It is possible that estrogen plays a role in the mechanism of life long ongoing bone adaptation. Bone response to mechanical stimuli is often described in terms of the previously discussed mechanostat, which predicts that bone responds to mechanical loading exceeding a specific upper threshold limit (causing bone formation) or falling under a the lower threshold limit, and this adaptation occurs so that strains remain within certain limits[9]. Frost hypothesized that estrogen alters the set point of the mechanostat by increasing the sensing of strain signals. Thus the bone cells would sense a higher strain than there actually is and initiate bone formation. The loss of estrogen production at menopause is associated with a marked increase in bone remodeling caused by an increase in bone formation and resorption in each BMU. However, unlike during the premenopausal years, when the rates of formation and resorption are coupled, the rates are disproportionate, with resorption exceeding formation, and thus resulting in net bone loss. Frost suggested that estrogen depletion resets the mechanostat to a lower set-point, causing an “erroneously perceived disuse,” even if magnitude of loading strain is kept the same[9]. Resetting the lower set-point should mainly

cause net bone loss from endocortical and trabecular surfaces where mechanical strains are least. Indeed, we see bone loss (declining cross-sectional area through increased endocortical expansion) only among postmenopausal nonusers of estrogen replacement therapy (ERT). Along with bone loss, we increased subperiosteal apposition to maintain bone strength. This would seem to be consistent with Frost's conjecture on the effects of estrogen depletion[33]. However, though circulating estrogen has a positive effect on bone density, there is a lack of evidence in human studies indicating that estrogen replacement has an anabolic effect on bone[16]. Thus while those who do not use ERT show increased CSA and decreased bone density, those who use ERT do not necessarily show increased bone formation. There is also a lack of longitudinal data of changes in bone structure and strength in women using ERT.

The mechanism of estrogen on reducing bone turnover is complex and involves interaction with many cells and regulation of several mediators[27]. At the cellular level, estrogen may influence bone growth by controlling production of cytokines, growth factors and prostaglandin from hematopoietic linage cells and mesenchymal lineage cells in the marrow[27, 216]. Specifically, estrogen suppresses levels of cytokines (interleukin-1 and -6 and tumor necrosis factor alpha), which are osteoclastic precursors, and specific growth factors (type I collagen and transforming growth factor-beta), which are osteoblastic precursors. The reduction of osteoclasts and osteoblasts precursors directly affects osteoclasts development, activity and apoptosis [216]. Through limiting this mechanism of bone resorption, estrogen maintains bone mass[28].

The skeletal effects of estrogen might also result from indirect actions. Estrogen may affect the parathyroid gland, gut or kidney217. For example, estrogen may change the set-point at which parathyroid hormone (PTH) responds to serum calcium which would promote bone mineralization by reducing bone turnover. PTH is secreted in response to falling serum calcium, which in turn stimulates bone resorption in order to maintain calcium homeostasis. Thus, the level of calcium needed in an estrogen-replete woman is lower than what is needed

in estrogen deficient women before bone resorption is stimulated. Also, estrogen may stimulate calcitonin release, which also limits bone turnover29.

However, recent research seems to suggest that the direct bone actions of estrogens are receptor mediated rather than indirect via the aforementioned secondary effects[218, 219]. Estrogen Receptors (ER) have been discovered to exist on the surfaces of osteoblasts, osteoclasts and osteocytes[220-224]**.** The two forms that have been identified are estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ), and each bone cell type contains both subtypes of receptors, but their distributions within bone differ[225]. It seems each type stimulates different responses on the cell surface and appear to compete against each other. ERα appears to enhance mechanically induced bone formation, while the ERβ receptor can be stimulatory, inhibitory or neutral[15, 22, 31, 226]. Much of the research looking at ERs is found in animal studies. Female ERA knockout (KO) mice are partially protected against ovariectomy induced loss of trabecular bone, which suggests that ERB can partially substitute for ERA, although higher does of estrogen are required[21]. ER significance on human bone has yet to be determined, thus further research on the ERs in humans along with the relationship between loading and circulating estrogen in premenopausal women is needed to further clarify and define possible prevention and treatment options of low bone mass and osteoporosis.

2.6.1.2 Progesterone's effects on bone remains controversial

Although the effects of estrogen on the skeleton are well documented, those of progesterone have received less attention by researchers. Consequently, the physiological importance of progesterone signaling in bone growth, turnover, and loss remains unclear[227, 228]. Studies of animals are limited, but seem to show that treatment with synthetic progesterone decreases bone resorption and increases formation[229]. This differs from estrogen, which seems to only decrease bone resorption. Naturally produced progesterone also seems to have a weak anabolic effect on the skeleton. In a study of ovariectomized rate (OVX), and those with pseudopregnancy (a condition of estrogen deficiency with elevated progesterone), having pseudopregnancy was not associated with cancellous bone loss in the rat, while the OVX had

significant bone loss[230]. Other studies even show increased bone formation. Studies in which progesterone was administered to OVX rodents at physiological concentrations reported increased periosteal bone formation, inhibited bone resorption, and enhanced estrogen-induced bone formation at endocortical surfaces[231, 232]. In other animal models, OVX rodents and dogs that were administered physiological concentrations of progesterone showed that progesterone seemed to prevent bone loss from multiple sites and in some cases actually stimulated new bone formation, particularly in cortical bone[233, 234].

Though it appears that progesterone promotes bone formation and/or increase bone turnover, it is possible, through estrogen-stimulated increased progesterone binding to the osteoblast receptor, that progesterone plays a role in the coupling of bone resorption with bone formation, similar to the effects of estrogen. A model of the interdependent actions of progesterone and estrogen on appropriately-"ready" cells in each bone multicellular unit can be tied into the integrated secretions of these hormones within the ovulatory cycle[227]. That is, the phases of the bone remodeling cycle may follow in parallel with temporal changes in gonadal steroids across the ovulatory cycle. Increasing estrogen production before ovulation may reverse the resorption occurring in a "sensitive" bone multicellular unit while gonadal steroid levels are low at the time of menstrual flow. The bone remodeling unit would then be ready to begin a phase of formation as progesterone levels peaked in the midluteal phase. From this perspective, the normal ovulatory cycle looks like a natural bone-activating, coherence cycle. More research is needed to explore the possible relationships between sex hormone changes during the menstrual cycle and the changes in the “cycle” of bone. Much further data are needed about the interrelationships between gonadal steroids and the "life cycle" of bone.

In studies of humans, which to date have been mostly focused on postmenopausal women, progesterone treatment indicates that it decreases the markers of bone resorption, but does not appear to elevate the markers of bone formation[235]. Progesterone was also reported to reduce cortical but not cancellous bone loss. In studies of combined estrogen and progestin treatment, there was both decreased resorption and increased formation, and combined

estrogen and progestin therapy was reported to be as effective as high-dose estrogen alone in preventing overall bone loss[236, 237].

Similar to the role of estrogen receptors, progesterone role in human bone metabolism may be affected at the cellular level by progesterone's physiological effect of binding to and activating the progesterone receptor (PR). Expression of PR in normal human osteoblast-like cells has been reported, and the level of PR expression in these cells can be stimulated by estrogen[220, 238]. Thus, it is possible that some of the effects on bone metabolism attributed to estrogen may be mediated by progesterone. Along with the direct effect of progesterone via binding to the PRs, it may also act indirectly through binding to glucocorticoid receptor and perhaps reducing the influence of glucocorticoids[239]. Glucocorticoids have been implicated in the process of bone loss through their ability to block 1,25-$(OH)_2$-vitamin D-induced osteocalcin synthesis[240].

However, not all studies report beneficial effects of progesterone on bone metabolism in postmenopausal women or animals. Some investigators have failed to demonstrate an effect of progesterone, with or without estrogen, on OVX-induced bone loss in rats[241, 242]. In women, more recent clinical trials dispute the earlier claims (discussed previously) that progesterone replacement therapy improves bone density in postmenopausal, premenopausal, OVX, or amenorrheic women[126, 243]. Future research may help explain these inconsistent findings.

3

RESEARCH METHODS AND STUDY DESIGN

3. RESEARCH METHODS AND STUDY DESIGN

3.1 Overview

Healthy eumenorrheic women (18-30 years old) were recruited and screened for participation in the Women in Steady Exercise Research study. All outcome measurements took place at between days 7 to 10 of the menstrual cycle. Luteal phase length (LPL) and menstrual cycle length (MCL) were averaged over two menstrual cycles.

3.2. Participant Description

Women aged 18 to 30 years of age who were eumenorrheic and not currently taking any hormonal contraceptives were recruited. Further eligibility criteria included:

- Self-reported menstrual cycle length of 26 to 32 days over the 2 months prior to entering the study
- Intact ovaries and uterus
- Nulliparous
- No history of gynecological problems (e.g. fibroids, endometriosis, polycystic ovary syndrome)
- No hormonal contraception use within the past year
- No medical conditions or medications that would prohibit participation in a vigorous program of weight bearing aerobic exercise or would negatively impact our ability to test our primary aims (e.g. fibromayalgia, chronic fatigue syndrome, metabolic disorders, recent cardiovascular event, orthopedic limitations, psychiatric disorders requiring anti-psychotic drugs)
- No uncontrolled hypertension (systolic blood pressure over 160 and/or diastolic blood pressure over 99)
- No history of cancer within the past 5 years, excepting non-melanoma skin cancers
- BMI must be at least 18 but not greater than 40 (to avoid anorexic and morbidly obese participants for whom bone outcomes may be significantly altered compared to healthy controls)
- Weight stable (no changes $\geq$ 10% over the past year)

- Not currently or recently (past 6 months) pregnant
- Not planning to become pregnant during the study period
- Non-smokers (for at least the past year)
- Not more than 7 alcoholic beverages per week (alcohol alters estrogen metabolism)
- Sedentary (no exercise 3 times weekly or more over the past 6 months)
- Not planning to move away from the Twin Cities area during the period of the study

3.3 Measurements

Estrogens

Participants visited the UMN GCRC on the 7th day of the menstrual cycle for measurements and to begin three 24-hour urine collections. All participants were asked to not engage in any moderate or higher intensity exercise, drink any alcohol, or alter their usual diet for 48 hours prior to the start of urine collections. All urine was collected for three consecutive 24 hour periods (7th-10th day of the menstrual cycle) in collection bottles with one gram of ascorbic acid per liter for preservation.

The participant's collection bottles were kept cold and 0.1% sodium azide was added before separating into aliquots. The urine was stored in a -70 degree freezer until analysis. The three 24-hour collections were pooled and the 72-hour aliquots analyzed. In the case of an incomplete urine collection (as judged by urinary creatinine values), that urine was not included in the pool

Urinary estrogen metabolites were analyzed by the gas chromatographic/mass spectrometric (GC/MS) methods of Fotsis and Adlercreutz[244], although modified by Kurzer colleagues[245]. These assays were performed by GC/MS analysis on a Hewlett Packard 5971A quadruple instrument, using selective ion monitoring, after multiple extractions and chromatographic separations. Deuterated internal standards for all estrogen metabolites were used.

The two estrogens analyzed and used in this study include the primary estrogens: estrone (E_1) and estradiol (E_2). The intra-assay variability from a previous pilot study were 8.5% and 5.5% respectively. The inter-assay variability for each metabolite in the same pilot study was 3.4% and11.0% respectively.

Ovulation and menstrual cycle characteristics

Participants were provided with a log on which to record characteristics of all menstrual cycles during study participation, as well as 4 9-day ovulation kits (OvuQuick, Conception Technologies, San Diego, CA) for assessment of the timing and occurrence of the luteal surge during study participation. The OvuQuick kit measures the luteal surge by Enzyme-Linked Immunosorbent Assays (ELISA) with 96% accuracy with home use. Luteal surge has been observed to coincide within one day of ovulation[246]. The menstrual characteristics log (available upon request) asked participants to record the date and approximate time (morning, afternoon, evening, or while sleeping) on which menstrual flow began and ended, as well as qualitative statement regarding whether the flow was the same as usual. If the flow seems different, the participant recorded how it seemed different (e.g. heaviness of flow or other symptoms). Further, the ovulation kit was used to detect the luteal surge during each menstrual cycle. The date on which the ovulation kit results indicated the luteal surge occurred was recorded on the menstrual cycle log as well. The luteal phase length was determined by subtracting the date the next menstrual cycle starts from the date the luteal surge was detected by the ovulation kit. Luteal phase length was used as a surrogate for exposure to the hormone progesterone.

Body composition

Body composition was measured by dual energy x-ray absorptiometry (DXA, Lunar Prodigy, Lunar Corp., Madison, WI). Total body scans were taken at the University of Minnesota's General Clinical Research Center (GCRC) and analyzed using manufacturer software (Lunar v 5.54) for total body lean and fat mass. All measurements were taken by certified technicians. Previous research has shown CV for DXA measured fat mass and lean mass to be 1.9% and 1.5% respectively 247 . A digital scale and a scale mounted stadiometer (Scale-

tronix 5005 stand-on digital scale, Scale-tronix, White Plains, NY), calibrated weekly, was used to measure weight (to the nearest 0.1 kg) and height (to the nearest 0.1 cm).

IGF axis variables:

For assessment of IGF-axis variables, plasma samples were stored at –70°C, then sent at study completion to Diagnostic Systems Laboratories reference laboratories (Webster, TX). Random samples were sent in duplicate to be tested for variation – the lab was blinded to which samples were sent in duplicate. Enzyme-Linked Immunosorbent Assays (ELISA) of IGF-I, IGFBP-1, -2, and -3 was performed at the reference labs of Diagnostic Systems Laboratories (Webster, TX). Samples were run with two standard controls, included in the kit for each analyte. The sensitivities for the assays for IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 were 0.03 ng/mL, 0.04 ng/mL, 0.15 ng/ml, and 0.25 ng/mL, respectively. The intra- and inter-assay coefficients of variation for IGF-I were: 6.5% at 167 ng/mL and 4.8% at 133 ng/mL, respectively; for IGFBP-1 were: 2.5% at 31 ng/mL and 6.8% at 30 ng/mL, respectively; for IGFBP-2 were: 2.9% at 7.3 ng/mL and 3.1% at 5.7 ng/mL respectively; and for IGFBP-3 were: 7.3% at 74 ng/mL and 8.2% at 66 ng/mL, respectively. All IGF-axis plasma assays will be performed in duplicate with the mean used for analysis; samples were batch processed to ensure the same reagent batch will be used for all assays for a given participant. For all assays, technicians were blinded to treatment status.

PQCT Measurements

Muscle cross-sectional area

We assessed muscle cross-sectional area (MCSA, mm2) at the 50% and 66% site of the tibia (proximal to the distal endplate of the tibia) from peripheral QCT measurements (Norland/Stratec XCT 3000 bone scanner; Stratec Medizintechnic GmbH, Pforzheim, Germany). We analyzed MCSA at these sites using Contour mode 1 (-100 mg/cm3), Peel mode 2 (40 mg/ cm3) and Cort mode 1 (710 mg/cm3) (Stratec software, Version 5.4). Scans will be excluded because of movement artifacts.

Bone Measurements

Peripheral QCT measurements will be used to acquire a single 2.3 ± 0.2 mm slice of the left tibia at the 4%, 50% and 66% sites, and the 4% and 50% site of the non-dominant radius. We used a voxel size of 0.5 mm and a scan speed of 30 mm/s. We acquired a 30 mm planar scout view over the joint line to define the anatomic reference line. The pQCT scans were analyzed using Cort mode 1 (710 mg/cm3). Measurements were made by one of three trained operators and a single operator will analyze all scans. Bone mineral content (BMC), bone geometry (total cross-sectional area, mean cortical cross-sectional wall thickness, cortical density, trabecular density), bone strength (CSMI and polar SSI) and muscle cross-sectional area will be assessed at the dominant tibia (1 scout scan and 3 slices, 4%, 50% and 66% of limb length) and the nondominant forearm (2 slices, 4% and 50% of limb length) through non-invasive Peripheral Quantitative Computed Tomography (pQCT). Scans will be excluded if participant movement caused blurred images or because of excess artifacts. A pQCT anthropomorphic phantom was scanned daily to maintain quality assurance. The scans will be performed at baseline and in a subgroup of women who return to be rescanned after 9-12 months of unsupervised physical activity or sedentary activity.

Bone–muscle strength indices

Bone measures from pQCT will be used to calculate bone strength for bending and compression: polar strength strain index (SSIp) for proximal (bending) sites and bone strength index (BSI) for distal (compression) sites. For both indices, we used MCSA as a surrogate for muscle force. Bone strength in compression is dependent on bone cross-sectional area; therefore, we used the ratio of CoA to MCSA as a BSI for compression at the tibial shaft. Bone strength in bending is dependent both on the section modulus and the length of the lever arm. Therefore, we calculated a bone strength index (SSIp) as [section modulus / (tibial length / 2)].

Other assessments

Dietary assessment

To assess usual food and nutrient intake the DHQ, a food frequency questionnaire[248] was administered by interview at the first clinic visit. The staff who administered the dietary

survey was blinded to treatment status. Furthermore, these dietary measurements provided an opportunity to expand our less than complete knowledge regarding the effect of exercise training on dietary habits.

Submaximal aerobic fitness

An assessment of sub-maximal fitness was performed using a treadmill at the Laboratory of Physiological Hygiene and Exercise Science (LPHES) at the University of Minnesota. After a five minute warm-up, participants walked on the treadmill at a steady speed (3.5 miles per hour), and the percent grade on the treadmill was be increased two percent every two minutes until the participants reach 80 percent of their age-predicted maximum heart rate (max HR), defined as 220 – age. If participants were within ten beats of their 80% MHR, incline was increased by only one percent every two minutes until completion of the assessment. Heart rate during this test will be measured using Polar Heart Rate monitors (Polar Electro Inc., Woodbury, NY). This workload was converted into Metabolic Equivalents (MET) using a standard conversion formula. The trainers that performed these tests were blinded to treatment status. After the fitness assessment, participants were informed of their group assignment (control or treatment).

3.4 Statistical Data Analysis

After data is cleaned, descriptive measures including counts of women present and missing, the means (medians) and standard deviations of baseline data will be generated. Baseline distributions will be examined for outliers and skewness. Stepwise linear regression will be used to determine which factors are the highest predictors of variance in bone measures. Women will than be separated into tertiles based on estrogen levels and lean mass levels, and each group will be compared to each other to determine the relationship between loading and estrogen. One-way ANOVAs will be used to test for significant differences in independent variables (bone measures, loading measures, body composition and lifestyle factors) among the groups. Significance will be set at $p<0.05$. See Table 3-1.

Table 3-1. 3 x 3 table used for ANOVA and ANCOVA analyses

	Low Estrogen (-)	Medium Estrogen(/)	High Estrogen (+)
Small Load (-)	- -	- /	- +
Medium Load (/)	/ -	/ /	/ +
Large Load (+)	+ -	+ /	+ +

Interaction between load and estrogen levels will be assessed using analysis of covariance (ANCOVA) for repeated measures. If the significance of the group × factor interaction was $p < 0.10$, the effect will be localized utilizing simple contrasts. The level of statistical significance chosen for the contrasts was $p < 0.05$. One-way ANCOVA was used to independent effects of load and estrogen on measures of bone vBMD, geometry and strength. Because it is statistically not possible to control for measurement position errors, we excluded those scans in which the total CSA (including bone marrow) changed by >10% at the proximal femur. The reason for excluding these scans is that it is physiologically not possible for a healthy woman to have a change in total CSA of >10% during a 1 year period.

3.5. Limitations and Strengths of Proposed Study

3.5.1. Limitations

This study's major limitation is the cross-sectional nature of the study. We can not determine any causal effects of the variables entered into our models, thus this research serves as an exploratory study to help us determine future research avenues.

3.5.2 Strengths

As far as we know, this is the first study to explore the characteristics of the independent and/or interactive effects of estrogen and mechanical loading on the human skeletons, along with the only study to look at predictors of bone strength in healthy sedentary premenopausal women using the wide variety of variables available to us (specifically, urinary measures of estrogens and luteal phase lengths as determined by ovulation testing). Also, measuring bone variables with the pQCT will provide much needed data on structure and strength of the bone in premenopausal women, which is currently missing in the literature. As previously mentioned, DXA allows for only a 2-D image to be produced, which limits the accuracy of the actual shape of the bone.

Also providing strength to the study is the use of dietary records and past physical activity interviews, which will allow us to control for other variables that have been suggested to also interact with loading and/or estrogen (exercise during adolescence, Vitamin D, Calcium and protein intake, etc).

4

MANUSCRIPTS

Bone strength and geometry in sedentary premenopausal women is primary predicted by measures of muscle load, estrogen and menstrual cycle characteristics

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ABSTRACT: Mechanical loading and sex steroids are hypothesized to play an important role in bone metabolism and to influence bone health in premenopausal women. PURPOSE: To determine predictors of bone strength, geometry and volumetric bone mineral density (vBMD) in sedentary premenopausal women. METHODS: Baseline data was used from a subset of healthy, sedentary, premenopausal women (n = 67, aged 18-30 years; mean BMI 23.96±3.1) enrolled in a study of physical activity and ovulation (WISER). Estrogen status was determined by 3-day urine collection. Menstrual cycle length (MCL) and luteal phase length (LPL) were assessed by questionnaire and confirmed with ovulation kits over 2 menstrual cycles. LPL was used as a surrogate of progesterone exposure. Total body lean mass (LM) and fat mass (FM) were measured by dual energy x-ray absorptiometry (DXA). Volumetric bone mineral density (vBMD), bone geometry (total and cortical area), estimated bone strength (polar strength strain index, SSIp, and bone strength index, BSI) and muscle cross-sectional area (MCSA) were assessed by pQCT (XCT 3000) at the distal (4, 8%) sites and midshaft (50, 66%) sites of the tibia and radius. Trabecular and total vBMD, total area and BSI were reported for distal sites; cortical vBMD, total and cortical area, and SSI were reported for midshaft sites. Linear regression, adjusted for limb length and age, was used to determine predictors of bone variables. RESULTS: The primary determinant of SSI and geometry at the midshaft tibia was LM while FM was the primary predictor of vBMD ($p<0.01$). At the distal tibia, LM was the primary determinant of BSI, bone geometry, and vBMD ($p<0.01$). MCSA was the primary determinant of both bone strength and geometry of the radius midshaft, while LPL predicted vBMD ($p<0.01$). At the distal radius, menstrual cycle characteristics, MCSA and LM were the primary predictors of all bone variables ($p<0.05$). CONCLUSION: Since sedentary premenopausal women likely have lower MCSA and LM compared to active women, these data suggest bone health may be compromised in this population. Also, luteal phase length and menstrual cycle length may significantly affect bone health. Thus, less muscle along with subtle alterations in menstrual cycle characteristics may lead to compromised bone strength in sedentary women.

INTRODUCTION

Osteoporosis and consequential fractures are major public health concerns, with up to 60% of women suffering an osteoporotic fracture in their lifetime[1, 2]. It is therefore important to identify predictors and modifiable risk factors of osteoporosis to aid in development of prevention and treatment regimens. Several factors are thought to influence bone strength including genetics, mechanical loading, sex steroids and nutrition[3-7]. The mechanostat[8] and related theories of bone functional adapation[9-12] suggest that mechanical loading should primarily influence skeletal rigidity, while sex steroids and hormones should primarily influence bone mass (minerals) that can easily be released for physiological needs[13, 14].

In support of these theories, studies in both adolescent and post-menopausal women show that surrogates of mechanical loading such as muscle cross-sectional area (MCSA), lean body mass, or levels of physical activity are the strongest predictors of bone geometry and estimates of bone strength[15-17], while hormonal and nutritional factors appear to influence bone response to loading[6, 14, 18-23]. Although cross-sectional and prospective studies show that there is a positive association between lean mass and BMD in premenopausal women[24, 25], the associations between mechanical loading, hormonal and nutritional factors and bone strength in the young adult population have not been widely explored.

Sex steroids and growth hormones also are known to effect bone properties, with estrogen, progesterone, insulin-like growth factor (IGF) and IGF binding proteins (IGFBPs) playing significant roles in female bone development[26-29]. Studies suggest that premenopausal women with greater levels of circulating progesterone and estrogen have greater vBMD compared to women with lower levels of these circulating sex steroids[30, 31]. However, the extra vBMD is found mostly on the inside (endocortical) surface of the bone, which may not significantly increase bone strength[32].

Overall, there is a lack of data from studies that have comprehensively assessed hormonal, nutritional, and mechanical factors that may influence bone volumetric density, geometry and strength in young adult women. Therefore, the primary purpose of this study is to explore

predictors of bone geometry and strength in sedentary young adult women. Based on the mechanostat theory and evidence from animal studies, we hypothesized that 1) bone geometry and bending strength would be predicted primarily by estimates of mechanical load (as represented by lean mass or muscle CSA), and 2) other factors including sex steroids, and nutrition would predict bone volumetric density, especially in the unloaded radial sites.

METHODS

Participants

Healthy eumenorrheic women aged 18-30 years who were not currently taking any hormonal contraceptives were recruited to participate in the Women in Steady Exercise Research (WISER) study in Minneapolis, MN. Participants included in this analysis were recruited during the first 6 months of the five-year longitudinal study. Interested participants were required to attend an information session detailing the study, after which informed consent was obtained from women interested and eligible to participate. Further eligibility criteria included: Self-reported menstrual cycle length of 26 to 32 days over the 2 months prior to entering the study, no history of gynecological problems, no hormonal contraception use within the past year, no medical conditions or medications that could affect study participation or study outcomes, BMI 18 -- 40, weight stable (no changes > 10% over the past year), not currently or recently (past 6 months) pregnant, non-smokers (for at least the past year), not more than 7 alcoholic beverages per week, and sedentary (no exercise more than 2 times weekly over the past 6 months). Of the 78 women who participated in the first 6 months of the WISER study, 67 women agreed to participate in this sub-study to assess bone geometry and strength using peripheral quantitative computed tomography (pQCT).

Hormone and Menstrual Cycle Measurements

Participants visited the UMN GCRC on the 7th day of the menstrual cycle to begin three 24-hour urine collections. All participants were asked not to engage in any moderate or higher intensity exercise, drink any alcohol, or alter their usual diet for 48 hours prior to the start of urine collections. All urine was collected for three consecutive 24 hour periods (7th-10th day of the menstrual cycle) in collection bottles with one gram of ascorbic acid per liter for preservation as described by Aldercreutz[33]. The participant's collection bottles were kept cold

and 0.1% sodium azide was added before separating into aliquots. The urine was stored in a -70 degree freezer until analysis. The three 24-hour collections were pooled and the 72-hour aliquots analyzed.

Estrogens

Urinary estrogen metabolites were analyzed by the gas chromatographic/mass spectrometric (GC/MS) methods described elsewhere[34], although modified by the WISER laboratory[35]. These assays were performed by GC/MS analysis on a Hewlett Packard 5971A quadrupole instrument, using selective ion monitoring, after multiple extractions and chromatographic separations. Deuterated internal standards for all estrogen metabolites were used. The two urinary estrogens metabolites used in our analysis include estrone (E1) and estradiol (E2).

Ovulation and menstrual cycle characteristics

Participants were provided with a log on which to record characteristics of all menstrual cycles during study participation, as well as four 9-day ovulation kits (OvuQuick, Conception Technologies, San Diego, CA) for assessment of the timing and occurrence of the luteal surge during study participation. The OvuQuick kit measures the luteal surge by Enzyme-Linked Immunosorbent Assays (ELISA) with 96% accuracy with home use. Luteal surge has been observed to coincide within one day of ovulation[36]. The menstrual characteristics logs asked participants to record the date and approximate time (morning, afternoon, evening, or while sleeping) on which menstrual flow began and ended, as well as qualitative statement regarding whether the flow was the same as usual. If the flow seems different, the participant recorded how it seemed different (e.g. heaviness of flow or other symptoms). Menstrual cycle length (MCL) was entered into the analysis as another measure of estrogen exposure.

In addition, the date on which the ovulation kit results indicated the luteal surge has occurred was recorded on the menstrual cycle log. The luteal phase length (LPL) was determined by subtracting the date the next menstrual cycle starts from the date the luteal surge was detected by the ovulation kit. LPL was used as a surrogate for exposure to the hormone

progesterone.

Bone and Muscle Measurements

To obtain measures of bone density and geometry, slices (2.3 ± 0.2 mm) of the left tibia and were obtained at the 4, 8, 50 and 66% sites of the tibia and the 4, 8, and 50% sites of the non-dominant radius proximal to the distal plafond of the tibia and radius using peripheral quantitative computed tomography (pQCT, Norland/Stratec XCT 3000 bone scanner, Stratec Medizintechnic GmbH, Pforzheim, Germany). A voxel size of 0.4 mm was used and the scan speed was set at 25 mm/s. The anatomic reference line was determined by acquisition of a 30 mm planar scout view of the joint line. The distal sites of the tibia and radius were assessed for trabecular volumetric density (TrabDen, mg/mm^3), total volumetric density (ToD, mg/mm^3), and total bone cross-sectional area (ToA, mm^2) using Contour mode 3 (200 mg/cm^3), Peel mode 5 (automatic), and Cort mode 3 (169 mg/cm^3). An estimate of the bone strength (Bone Strength Index, BSI) was calculated as ToA * ToD^2 [28, 29]. The proximal sites were assessed for total bone area (ToA, mm^2), total cortical area (CoA, mm^2), cortical volumetric density (CoD, mg/mm^3), and polar strength-strain index (SSIp, mm^3) using Contour mode 1 (710 mg/cm^3), Peel mode 2 (540 mg/cm^3), and Cort mode 1 (480 mg/cm^3). Threshold values and cort/peel modes were set according to manufactures recommendations. Measurements were made by one of three trained operators and a single operator will analyze all scans. A pQCT anthropomorphic phantom was scanned daily to maintain quality assurance.

Muscle cross-sectional area (muscle CSA, cm^2) was also determined at the 50 and 66% site of the tibia and 50% site of the radius. One of three trained operators performed the measurements and one operator analyzed all scans. Precision with repositioning was determined in our laboratory in adults (women n = 11, men n = 4, age 28.5 ± 6.5 years) as a coefficient of variation (CV, %) and varied from 0.28 (ToDen) to 1.20 (TrabA) at the distal tibia and from 0.31 (CoDen) to 0.41 (ToA) at the shaft. An anthropomorphic phantom was scanned daily for quality assurance.

Body Composition

Body composition was measured by dual energy x-ray absorptiometry (DXA, Lunar Prodigy, Lunar Corp., Madison, WI). Total body scans were taken at the University of Minnesota's General Clinical Research Center (GCRC) and analyzed using manufacturer software (Lunar v 5.54) for total body lean and fat mass. All measurements were taken by certified technicians. Previous research has shown CV for DXA measured fat mass and lean mass to be 1.9% and 1.5% respectively[37]. A digital scale and a scale mounted stadiometer (Scale-tronix 5005 stand-on digital scale, Scale-tronix, White Plains, NY), calibrated weekly, was used to measure weight (to the nearest 0.1 kg) and height (to the nearest 0.1 cm).

Dietary assessment

To assess usual food and nutrient the Diet Health Questionnaire (DHQ), a food frequency questionnaire[35] was administered by interview at the first clinic visit. Study staff who administered the dietary survey were blinded to treatment status. Dietary variables were assessed using Diet*Calc version 1.4.3 (2005) software and analyzed for total calorie, macronutrient and micronutrient intake. We used calcium, vitamin D, and protein in the regression analysis.

Statistical Analysis

Data were analyzed using SPSS software (version 13.0; SPSS Inc., Chicago, IL). Scatterplots were used to identify outliers and any points that were at least 3.0 SD away from the group mean were excluded (n=3). Histograms were created to check for the normality of the data. Descriptive characteristics are reported as means, standard deviations (SD) and 95% confidence intervals (CI). Linear regression analyses were used to identify significant predictors of bone geometry and strength variables using stepwise selection. Height (tibia) , muscle CSA (for radius) and age was forced entered in the first step in every case adjust for size and age. After height/muscle CSA and age, other variables considered in the initial model included lean mass, fat mass, calcium, vitamin D, protein, estradiol (E2), estrone (E1), average luteal phase length (LPL), average menstrual cycle length (MCL) and ethnicity. For the regression models, we report the regression Beta (β) value and standard error (SE), *r*-

squared, and p-value. Variables were included in the final models if P < 0.10 and overall models were considered significant if model P < 0.05.

RESULTS

Descriptive characteristics

Descriptive characteristics of the population are reported in Table 1. Participants ranged in age from 19-31yrs, had BMI values ranging from 17.74-40.55, and percent fat ranging from 20.57-54.31. Total calcium (911.80 $\pm$ 458.25), and percent calories from fat (23.38-45.76%) were within or slightly above recommended amounts for young adults. Total caloric intake (2240.88 $\pm$ 502.29) was slightly above USDA recommended intake for sedentary young women.

Table 4-1. Descrpitive characteristics of sedentary women aged 18-30

	N	Mean (SD)
Age (years)	64	25.66 (3.11)
Height (cm)	64	164.94 (7.95)
Weight (kg)	64	65.18 (14.32)
BMI	64	23.89 (4.52)
Body fat (%)	64	34.81 (7.79)
Lean (kg)	64	39.31 (5.37)
LPL (days)[a]	61	13.18 (2.11)
MCL (days)	58	29.95 (4.90)
Calc (mg)	52	911.80 (458.25)
Estradiol (ng/mL)[b]	64	61.56 (19.21)
Estrone-S (ng/mL)[b]	64	2.50 (0.86)

[a]Luteal phase length is based on time between ovulation and onset of menses

[b]Urinary estrogen measures are averaged from days 7-9 of menstural cycle

Bone density, geometry and strength

Absolute values of bone geometry and strength (mean, 95% CI) are shown in Table 4-2. There are currently no reference data for pQCT bone outcomes. Rauch and colleagues found pQCT measures at the distal radius that were not significantly different from our measures[38, 39]. Until recently, pQCT measures of the tibia were not able to be performed, thus

comparable data for all measures is not available. However, our measures of distal and proximal tibia area and density agree with a recent study of bone structural characteristics in young women[40, 41].

Table 4-2. Absolute values of bone geometry and strength

Tibia pQCT Bone Outcomes in Sedentary Women aged 18-30

Tibia		N	Mean (SD)	95% CI
4%	ToDen (mg/mm³)	64	317.46 (45.80)	304.17, 330.76
	TrabDen (mg/mm³)	64	251.60 (38.99)	240.28, 262.92
	ToA (mm²)	64	902.80 (107.53)	871.58, 934.03
	BSI (mg²/mm⁴)	64	259.99 (60.89)	242.31, 277.67
50%	CoDen (mg/mm³)	64	1178.67 (26.91)	1171.89, 1185.45
	ToA (mm²)	64	397.35 (51.97)	384.27, 410.44
	CoA (mm²)	64	283.24 (41.35)	272.83, 293.66
	SSIp (mm³)	64	1697.75 (315.93)	1618.18, 1777.32
66%	CoDen (mg/mm³)	64	1147.74 (25.97)	1141.14, 1154.33
	ToA (mm²)	64	516.22 (71.54)	498.05, 534.39
	CoA (mm²)	64	279.95 (39.29)	269.97, 289.93
	SSIp (mm³)	64	2207.82 (399.11)	2106.46, 2309.17
Radius		**N**	**Mean**	**95% CI**
4%	ToDen (mg/mm³)	52	386.52 (65.27)	368.35, 404.69
	TrabDen (mg/mm³)	52	210.35 (38.17)	199.72, 220.97
	ToA (mm²)	52	258.43 (42.65)	246.56, 270.30
	BSI (mg²/mm⁴)	52	383.38 (104.23)	354.37, 412.40
50%	CoDen (mg/mm³)	52	1203.51 (21.24)	1198.20, 1208.81
	ToA (mm²)	52	100.21 (15.59)	96.32, 104.11
	CoA (mm²)	52	80.66 (11.66)	77.74, 83.57
	SSIp (mm³)	52	207.88 (49.32)	195.56, 220.20

Presented as estimated marginal mean (SD) and 95%CI

ToA = total area; CoA = cortical area; ToDen = total density; CoDen = cortical density; TrabDen = trabecular density; SSIp = polar strength-strain index; BSI = bone strength index

Tibia. Beta values, p-values and r^2 values for regression models at the tibia are reported in Table 4-3. (distal tibia) and Table 4-4. (proximal tibia). For all models, age and height were forced enter. After height and age, lean mass was the primary predictor of BSI (r^2 = 0.42, P < 0.01), luteal phase length (LPL) was the primary predictor of 50% SSIp (r^2 = 0.29, P<0.01), and lean mass primarily predicted 66% SSIp (r^2 = 0.58, P<0.01). Only lean mass explained the variance in trabecular and total density (vBMD) at the 4% tibia site with the total model explaining 12% and 8% respectively (P < 0.05), while total area was predicted by lean mass and vitamin D (r^2 = 0.35, P < 0.01). Lean mass was also the only predictor after age and height of total area (r^2 = 0.62, P < 0.001) and was the primary predictor of cortical area, explaining 58.6 % of the total model explanation of 61.8% (P < 0.01). Cortical density at the 50% site was inversely predicted by fat mass (r^2 = 0.20, P < 0.01). At the 66% site, lean mass was the primary predictors of total area and cortical area (P < 0.01), explaining 48-58% of the total model. Fat mass was inversely predictive of cortical density (r^2 = 0.15, P < 0.01) and with lean mass, explained 19% of the cortical density (P < 0.01)

At the tibia, measures of muscle load were the primary predictor of bone variables at the tibia. Menstrual cycle characteristics explained 3-8% and urinary measures of estrone explained 3-7% of the variance in geometric and strength measures.

Table 4-3. Stepwise Regression analyses for predictors of bone geometry and strength variables of the distal tibia in sedentary women aged 18-30

	4% Tibia			
		Standarized Beta	*P* Value[b]	Model r^{2c}
Trabecular Density (mg/mm³)	Age (yrs)[d]	-0.15800	0.193	
	Height (cm)	-0.48000	0.011	-0.021
	Lean mass (kg)	0.59400	0.001	0.117*
Total Density (mg/mm³)	Age (yrs)	-0.09500	0.442	
	Height (cm)	-0.42200	0.027	-0.029
	Lean mass (kg)	0.53400	0.005	0.079*
Total Area (mm²)	Age (yrs)	-0.12000	0.253	
	Height (cm)	0.21300	0.179	0.247**

	Vitamin D (µg)	-0.26300	0.011	0.307**
	Lean mass (kg)	0.35600	0.023	0.353**
BSI (mg²/mm⁴)	Age (yrs)	-0.10400	0.287	
	Height (cm)	0.03800	0.798	0.264**
	Lean mass (kg)	0.62300	0.000	0.423**

[a]To control for body size, age and height were force-entered into the first step regardless of their significance in the overall model

[b]The P value listed here is based on the F test for the individual variable entry to the final model.

*Significance of overall model, P<0.05

**Significance of overall model, P<0.01

Table 4-4. Stepwise Regression analyses for predictors of bone geometry and strength variables of the proximal tibia in sedentary women aged 18-30

Table 4
Stepwise Regression analyses for predictors of bone geometry and strength variables of the proximal tibia in sedentary women aged 18-30

	66% Tibia				**50% Tibia**			
		Standarized Beta	*P* Value[b]	Model r^{2c}		Standarized Beta	*P* Value[b]	Model r^{2c}
Cortical Density	Age (yrs)	0.05300	0.656		Age (yrs)[a]	0.04000	0.727	
(mg/mm³)	Height (cm)	0.28100	0.114	-0.015	Height (cm)[a]	-0.13700	0.237	0.013
	Fat mass (kg)	-0.31100	0.011	0.151**	Fat mass (kg)	-0.44400	0.000	0.196**
	Lean mass (kg)	-0.378	0.04	0.192**				
Total Area	Age (yrs)	-0.27100	0.001		Age (yrs)	-0.17900	0.026	
(mm²)	Height (cm)	0.13600	0.248	0.416**	Height (cm)	0.00500	0.964	0.378**
	Lean mass (kg)	0.59000	0.000	0.575**	Lean mass (kg)	0.76100	0.000	0.622**
	Estrone-S (ng/ml)	0.269	0.001	0.627**				
Cortical Area	Age (yrs)	-0.04300	0.621		Age (yrs)	-0.07300	0.363	
(mm²)	Height (cm)	-0.09200	0.488	0.21**	Height (cm)	-0.03200	0.795	0.287**
	Lean mass (kg)	0.78400	0.000	0.485**	Lean mass (kg)	0.71700	0.000	0.586**
	Menstrual cycle (d)	0.23500	0.007	0.527**	Fat mass (kg)	0.21200	0.014	0.618**
	Estrone-S (ng/ml)	0.186	0.031	0.555**				
SSIp (mm³)	Age (yrs)	-0.22200	0.003		Age (yrs)	-0.15200	0.160	

Height (cm)	0.10500	0.351	0.377**	Height (cm)	0.40900	0.000	0.214**
Lean mass (kg)	0.62900	0.000	0.579**	Luteal length (d)	-2.92000	0.007	0.29**
Estrone-S (ng/ml)	0.29400	0.000	0.653**				
Menstrual cycle (d)	0.17600	0.016	0.679**				

[a]To control for body size, age and height were force-entered into the first step regardless of their significance in the overall model
[b]The P value listed here is based on the F test for the individual variable entry to the final model.
[c]The r2 value represents the amount of variance explained by the model with current and preceding variables included.
*Significance of overall model, $P < 0.05$

Radius. Beta values, p-values and r2 values for regression models at the radius are reported in Table 5. Age and radius length were forced into each model. After radius length and age, menstrual cycle length (MCL) was the primary predictor of BSI (r^2 = 0.142, P < 0.01) and MCSA was the primary predictor of SSIp (r^2 = 0.47, P<0.01). Only lean mass predicted total area of the 4% radius (r^2 = 0.192, P < 0.01), while no variables predicted vBMD. At the 50% site, MCSA was the primary predictor of total area, cortical area and with MCSA predicting between 46-47% of the variance (p<0.01). Cortical density at the 50% site was primarily predicted by luteal phase length (LPL) with fat mass contributing (r^2 = 0.105, P < 0.05). Other important variables that influenced radius bone variables included fat mass, protein, lean mass, MCL and LPL.

DISCUSSION

The findings from this study contribute to the understanding of the predictors of bone strength and geometry in young adult women. Our data suggest that 1) surrogates of mechanical load (lean mass or muscle CSA) are the primary predictors of bone strength estimates (measured by pQCT); 2) volumetric BMD (vBMD) is primarily predicted by body composition (lean mass or fat mass) at the tibial (loaded) sites and by menstrual cycle characteristics at the radial (unloaded) sites; and 3) nutrition explained only a small amount of the variance to bone outcomes. These findings are consistent with the hypothesis that mechanical loading is the primary predictor of bone geometry and strength and that other factors influence bone volumetric density and influence the geometric adaptation especially at unloaded sites.

Throughout the discussion, we use terms bending and compression strength to refer to measures of polar strength strain index (SSIp) and bone strength index (BSI) respectively. Measures that influence SSIp and BSI include vBMD and bone geometry as represented by cross-sectional total area and cortical area (ToA and CoA respectively).

Mechanical loading is the primary predictor of bone geometry and strength

In this population of sedentary women, surrogates of muscle force (total body lean mass and muscle CSA) were the primary predictors of bone strength after accounting for body size and

age. Muscle force is responsible for a large portion of the load on bone and recent work suggests the importance of measuring MCSA to use as a surrogate for force rather than body weight[42]. We therefore used MCSA as a surrogate for muscle force, along with the more widely used measure of total body lean mass to further explore the role of muscle in bone adaptation. In accordance with the Mechanostat and other related theories of functional adaptation[11, 15, 19], our data suggest that the muscle load on a bone is associated with a beneficial adaptation of bone structure and bone strength. We found lean mass or MCSA to be the primary and most significant predictor of all the radial and tibial geometry and strength variables, and of trabecular vBMD of the tibia ($p<0.01$). These results provide evidence that premenopausal women may have a window of opportunity to increase bone strength and vBMD prior to menopause through changes in muscle load. A recent study showed a 2.5% increase in bone strength in women who participated in a 12-month high intensity jumping intervention, and the increase in strength was likely a result of a small but significant increase in periosteal circumference[43]. Female athletes show significantly greater bone geometry and strength in loaded bones[44] and that changes in bone geometry and strength as a result of mechanical loading have been shown to be maintained for at least 5 years[45]. Longitudinal studies could provide us with evidence of long lasting maintenance of positive bone adaptations.

Sex steroids and menstrual cycle characteristics effects on bone

Evidence suggests that estrogen inhibits bone turnover as well as possibly influencing the mechanostat via altering the response of bone to mechanical stimuli through surface-dependent locations of alpha and beta estrogen receptors[22, 27, 46, 47]. It has been proposed that estradiol is primarily responsible for endosteal apposition (or reduced formation)[18, 48]. Given its inhibitory role in bone turnover, estrogen should theoretically be positively associated with volumetric bone density and is thought to be negatively associated with periosteal circumference (as measured by total bone area). However, our data showed that estrogen was negatively associated with cortical density and positively associated with bone geometry and bending strength at the midshaft of tibia. Furthermore, estrogen was not related to trabecular bone outcomes in the distal tibia. Although these findings are conflicting

with existing evidence, it is of note that the participants of this study were all sedentary and had normal ovulatory menstrual cycles lengths, thus it may be that estrogen levels may need to significant above or below normal levels before its effects on bone can be determined.

Our data showed that after measures of lean mass, estrone explained 3-7% of the variance in bone bending strength and in tibial bone geometry, through increased total and cortical area. Others have found estrogen to be inversely predictive of bone subperiosteal width along with a positive association with endosteal diameter and cortical thickness in young women[49]. Similarly, in a study of postmenopausal women serum estradiol level was found to be inversely correlated with change in the periosteal diameter (r = –0.25, P=0.009) and positively correlated with change in bone mineral density (r = 0.34, P<0.001), suggesting that decreased estradiol levels are associated with increased periosteal bone apposition[50]. However, in our premenopausal population, our findings were inconsistent with these findings, as we found estrone to be positively predictive of tibial total area (50 and 66% site)suggesting increased periosteal diameter, but similar to these previous studies, we did find estrone to be positively associated with cortical area (66% site), suggesting an increased cortical width. Evidence from animal data suggest that estrogen may have dual effects on the bone response to loading by both stimulating bone formation on the endocortical surface and inhibiting periosteal expansion[23, 47, 51]. The effect on periosteal expansion could be either a direct effect through estrogen receptor alpha[10] or an indirect effect through increasing bone mineral mass that results in increased bone rigidity[18]. Our data suggest that estrone has a positive effect on both surfaces, and given the significant role of lean mass in our models, it may be that estrone has a permissive effect on loading induced periosteal formation. Also, measures of estrone were collected during a the mid-follicular phase of the menstrual cycle, when estrogen levels are low, and thus peak estrone levels were not known. It has also been shown that among regularly cycling women, there is considerable normal variability in the phases of the menstrual cycle and that the follicular phase contributes most to this variability[52]. Longitudinal collection of estrogen measures may be needed to better understand the role of estrone in bone adaptation

The use of menstrual cycle characteristics can help explain effects of length of sex steroid exposure over the course of a cycle. We used luteal phase length (LPL) as a surrogate for progesterone exposure since women are exposed to the highest levels of progesterone during the luteal phase of the menstrual cycle and we used menstrual cycle length as another measure of estrogen exposure in conjunction with the 3-day urinary estrogen measures. While the women in this study were eumenorrheic (10-12 menstrual cycles per year), the subtle changes in differences in menstrual cycle length provide a surrogate for the LPL or MCL could cause women to be exposed to higher or lower amounts of progesterone and estrogen different levels of estrogen exposure. Those with longer LPLs have possibly more regular progesterone exposure than those with shorter LPLs. Though the role of progesterone in bone growth and metabolism is not as well understood, research suggests it behaves similarly to estrogen through stimulating increased bone formation on the endosteal surface and decreased bone turnover. Animal studies have shown a synergistic positive effect on bone when estrogen and progesterone are together added to bone cell culture[31, 53, 54] and others have found that the administration of progesterone to growing and aged rats that had undergone ovariectomy was associated with enhanced bone formation[55, 56]. Though the human data on progesterone effect's on bone remains controversial[57], evidence suggests that progesterone contributes to skeletal development and maintenance of bone mass in the adult premenopausal woman[58]. Our findings support the current evidence of positive relationship between bone strength and structure and progesterone exposure. We found LPL to predict 6% of cortical density the 50% radius. However, we also found LPL to be negatively associated with SSIp of at the 50% tibia. It may be that progesterone has a positive effect on density, but inhibits geometrical adaptation to loading needed to increase strength. The role of progesterone in the Mechanostat is not known and further exploration of LPL and other menstrual cycle characteristics may provide a better understanding.

Unlike the possible positive relationship between LPL and progesterone exposure, menstrual cycle length (MCL) is negatively correlated with total estrogen exposure. Studies find that estrogen concentrations were highest in women with shorter cycles[59]. Since estrogen stimulates bone formation on the inner surface, we see increases in bone density as

measured by DXA between normally menstruating women and those with abnormal cycles (olgimenorrhea or amenorrhea), however, pQCT data suggests that periosteal expansion is greater in women with decreased estrogen exposure but vBMD is decreased, as seen in postmenopausal women not on hormone therapy[60]. A recent study in eumenorrheic premenopausal women found that menstrual cycle length was inversely associated with whole-body BMD[61]. We found MCL to be positively predictive of cortical area and SSIp at the 66% tibia, while its only significant predictive value in the radius was at the BSI of the 4% site, predicting 5% of BSI ($p<0.01$). Since bone strength depends on a combination of bone density and bone area, the increased in periosteal expansion likely compensates for the decreased BMD. Our findings support this hypothesis. The longer MCL was predictive of increased cortical area and bone strength of the proximal tibia, along with bone strength of the distal radius. Thus, the decreased estrogen exposure experienced by women with longer MCLs may stimulate increased periosteal expansion through slightly increased bone turnover and increased periosteal bone formation leading to significant increased bone strength (as you will recall, small increases in periosteal bone formation creates significant increases in bone strength). Unlike in postmenopausal women, the levels of estrogen in the women in our study were still high enough to maintain vBMD since women in this study had to be eumenorrheic. Research shows women who are eumenorrheic have significantly greater BMD than women with olgimenorrhea (between 4-10 cycles/year) or amenorrhea (3 or less cycles per year, respectively)[61, 62] . Thus a slightly increased periosteal expansion along with maintained or slightly decreased vBMD contribute to gains in bone strength.

Fat mass negatively predicted bone density

We found that increased fat mass (FM) was associated with decreased volumetric BMD (vBMD). The role of FM in the premenopausal population is not well known. Several epidemiologic studies have reported that both fat mass may help to determine bone mass in postmenopausal women [63,64] and premenopausal women [65]. However, other researchers have found significantly negative relations between fat mass and bone mass for a given body weight [66-68]. All of these studies, however, used DXA to assess bone density, thus the

relationship of fat mass to pQCT assessed bone measurements in this population is not known.

The reasons we found a negative correlation between FM and vBMD could be one of several ideas. FM could be surrogate marker for lifestyle factors that are themselves negatively associated with BMD. Women who are physically active have greater lean mass and less fat mass compared to sedentary controls[69]. Also, women with greater amounts of fat mass may be at greater risk for cardiovascular disease, and epidemiologic evidence has linked osteoporosis and cardiovascular disease[70]. Greater fat mass could also indicate a nutritional intake higher of nutrients that seem to negatively affect bone. In one study, after adjustment for weight, fat mass, and other confounders, significantly negative relations were found between bone mass and cholesterol, and LDL concentrations[71, 72]. The mechanism of the reduction in bone mass may be explained by studies that have shown that oxidized lipids inhibit osteoblastic differentiation from preosteoblasts in vitro and bone formation in vivo[73, 74]. This inhibition of preosteoblast differentiation may result in reduced bone mineralization.

Nutritional intake influence on bone

The influence of nutrient intake on bone variables is still not well defined, studies suggest that there is both a positive relationship among calcium, vitamin D and bone health[75, 76] or no relationship[77, 78] in females. Although vitamin D and protein explained a small amount of the variance in 2 of the models (distal radius vBMD and cortical area at the proximal radius respectively), we found no consistent relationships between nutritional status and bone outcomes in this population. These data could suggest that there is relatively small (or no) major influence of nutrition on bone strength in young adult women. Notably, our sample is relatively homogenous and all participants reported consuming close to the recommended intake of most nutrients. Most nutrients are thought to have a threshold effect[79] on bone mass, and challenges exist in assessing dietary intake since it can be difficult collecting information on ingredients in dishes as well as in meals shared by family members, along with changes in nutrients due to cooking effects. Also important to consider is that assessing

only nutrient intake is inadequate, since other nutrients affect bioavailability. Overall, our data suggest a relatively small role for nutrition on bone health in this population.

Limitations

The cross sectional nature of the present study does not allow for determining causal relationships, and therefore, such theories explaining these observations remains to be investigated. It is also be possible that the measures of estrogen and menstrual cycle characteristics may be representing other hormones that influence bone metabolism including testosterone, growth hormones and growth factors.

Conclusions

The results of this study suggest that mechanical loading and hormonal milieu are important determinants of bone geometry and strength in young adult women. The use of the cross-sectional design of our study does not allow us to make any causal relationships, however these data provide strong supporting evidence for an important role of mechanical loading and sex steroids on bone strength. Longitudinal and intervention studies are needed to further explore these relationships in this population. Questions remain regarding if these skeletal adaptations remain into the peri- and postmenopausal years. If these positive changes are maintained into later adulthood, then women may be at a reduced risk of skeletal fragility during the peri- and postmenopausal years, when incidence and prevalence of osteoporosis are the greatest.

Estrogen and lean mass are independently associated with bone strength and geometry in premenopausal women.

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ABSTRACT: Mechanical loading and estrogen are known to effect bone geometry and strength. It is hypothesized that bone strength adapts to mechanical loading through lean mass, while estrogen is thought to increase the sensitivity of the bone to mechanical loading and to act directly on bone surfaces. PURPOSE: To explore the proposed independent and interaction effects between estrogen and lean mass on bone geometry and strength. METHODS: Baseline data was used from a subset of healthy, sedentary, premenopausal women (n = 67, aged 18-30 years; mean BMI 23.96±3.1) enrolled in a study of physical activity and ovulation (WISER). Estrogen status (estrone levels) was determined by 3-day urine collection. Total body lean mass (LM) and fat mass (FM) were measured by dual energy x-ray absorptiometry (DXA). Volumetric bone mineral density (vBMD), bone geometry (total and cortical area), estimated bone strength (polar strength strain index, SSIp, and bone strength index, BSI) and muscle cross-sectional area (MCSA) were assessed by pQCT (XCT 3000) at the distal (4%) sites and midshaft (50, 66%) sites of the tibia. Trabecular and total vBMD, total area and BSI were reported for distal sites; cortical vBMD, total and cortical area, and SSIp were reported for midshaft sites. Women were separated into tertiles of LM and estrone (E1). One-way ANOVA was used to determine differences in age, height, weight, E1, LM, calcium and menstrual cycle characteristics. ANCOVA was used to test for independent and interaction effects of E1 and LM, adjusted for age, height and weight. RESULTS: LM and E1 were independently associated with SSIp, cortical area and total area at the 66% site ($p<0.05$), and with cortical and total area at the 50% site ($p<0.05$). LM was also associated with vBMD at the 4% site ($p<0.05$). Though both LM and E1 had significant independent effects on bone geometry and strength, we did not find a significant interaction effect on any bone measures. However, the women in the highest tertiles for both LM and E1 had a 33% and 14.4% greater SSIp at the 66% and 50% sites, respectively. CONCLUSION: Young women's estrone levels and LM have important consequences for the development of young adult cross-sectional geometry and therefore, of bone strength. If low estrogen and LM levels reduce bone strength, specific populations may at risk for skeletal fragility, such as sedentary women or young women with amenorrhea, anorexia, or hypogonadism.

Introduction

The largest voluntary loads on bones come from muscles and bone strength adapts to theses loads via adding and/or removing bone through the mechanisms of modeling and remodeling. The physiological basis of the relationship between muscle function and the skeleton is illustrated by the mechanostat theory, which presumes that muscle contractions induce tension in the bone, which in turn activates bone modeling and remodeling[1]. According to the mechanostat, bone formation and bone resorption is stimulated by the local mechanical deformation of bone by peak forces caused by muscles. To adapt bone strength and mass to these forces, special strain threshold ranges determine where modeling adds and strengthens bone, and where remodeling conserves or removes it. Studies in both animals[2-4] and humans[5, 6] support this theory, with weight-bearing exercise or increases in lean mass being significantly associated with increased bone mass and bone area within the loaded bones, leading to increased bone strength. A study of fifty adult rats that were randomized into control or a 14-week progressive running program showed significant increases in bone mass and bone breaking load compared to sedentary controls[4]. In a human model, premenopausal women exposed to an endurance training intervention, 77% of bone density variability could be explained by the increase in total body lean mass[6]. In another study of young women, only lean mass was associated with bone bending and axial strength at the proximal femur[5].

Along with muscle load, the skeleton is a common target of the sex steroid estrogen. It is known that estrogen decreases bone turnover and increases endocortical contraction (packing of the bone on the inner surface, next to the marrow space, of bone), thus conserving bone mass[7]. Support for this concept often comes from studies of the effects of estrogen withdrawal on bone health. The occurrence of osteoporosis around the time of menopause suggests that estrogen deficiency results in increased levels of bone turnover[8], and ovariectomized rats demonstrate significant bone loss within the first couple months[9]. Estrogen may also play an important role in adaptation of bone to loading. Frost proposed that the sensitivity of bone to loading may be increased by the presence of estrogen, and therefore exercise may have a more beneficial effect on bone formation in the presence

rather than the absence of estrogen[10]. Studies in animal models show that variations in estrogen or estrogen receptor levels can affect osteogenic responses to mechanical loading[11-14], and research suggests that estrogen reduces the osteogenic response at the periosteal surface of bone but allows for increased response on the endocortical surface[15, 16]. Human data of estrogen's role in adaptation to loading is lacking. Previous research suggests independent effects of lean mass and estrogen on bone health, but it is not known if there exists an interaction effect of these two factors on bone health in healthy premenopausal women. If estrogen is an important component of reducing bone turnover and increasing bone density, and mechanical load is an important component of adapting bone geometry, than women with the highest estrogen levels and the greatest levels of lean mass (as a surrogate for mechanical load) should have the strongest adult bones. However, very few studies in humans have been designed to collect the necessary data to address the relationships among estrogen, lean mass and bone strength[17]. Many previous studies commonly used dual energy x-ray absorptiometry (DXA) to measure BMD as a surrogate for bone strength. However, DXA does not take into account bone geometry and thus can only approximately evaluate the relationship between the muscle and skeleton. Bone strength depends on both the amount of bone surface and distribution of the bone surface, neither which can accurately be assessed with DXA. The use of newer technology such as peripheral quantitative computed tomography (pQCT) allows us to measure volumetric bone densities and geometric variables that characterize the architecture of the bone, which gives us a more accurate measure of bone strength.

Thus, the purpose of this study is to examine the relationships of lean mass and estrogen on bone strength and geometry outcomes as measured by pQCT. Based on the Mechanostat theory, we hypothesized that lean mass will significantly and positively effect bone strength a geometry. Given previous research suggesting estrogens role in reducing bone turnover, we also hypothesize an independent effect of estrogen levels on bone strength and vBMD. According to the Mechanostat, estrogen is thought to increase the sensitivity of bone to loading, thus we hypothesize that there will exist a significant relationship between lean mass and estrone on bone outcome measurements of strength and geometry, with women having

the highest levels of both estrogen and lean mass having the greatest bone strength compared to women with the lowest levels of both variables.

Methods

Participant Description

Healthy eumenorrheic women aged 18-30 years who were not currently taking any hormonal contraceptives were recruited within the Women in Steady Exercise Research (WISER) study in Minneapolis, MN. The women were recruited during the first 6 months of the five-year longitudinal study. Interested participants were required to attend an information session detailing the study, after which informed consent was obtained from women interested and eligible to participate. Of the 78 women who participated in the first four months of the WISER study, 67 women had their bones scanned using the pQCT. Further eligibility criteria included: Self-reported menstrual cycle length of 26 to 32 days over the 2 months prior to entering the study, no history of gynecological problems, no hormonal contraception use within the past year, no medical conditions or medications that could affect study participation or study outcomes, BMI 18 -- 40, weight stable (no changes > 10% over the past year), not currently or recently (past 6 months) pregnant, non-smokers (for at least the past year), not more than 7 alcoholic beverages per week (alcohol alters estrogen metabolism), and sedentary (no exercise more than 2 times weekly over the past 6 months).

Estrogen

Participants visited the UMN GCRC on the 7th day of the menstrual cycle to begin three 24-hour urine collections. All participants were asked not to engage in any moderate or higher intensity exercise, drink any alcohol, or alter their usual diet for 48 hours prior to the start of urine collections. All urine was collected for three consecutive 24 hour periods (7th-10th day of the menstrual cycle) in collection bottles with one gram of ascorbic acid per liter for preservation as described by Fotsis and Aldercreutz[18]. The participant's collection bottles were kept cold and 0.1% sodium azide was added before separating into aliquots. The urine was stored in a -70 degree freezer until analysis. The three 24-hour collections were pooled and the 72-hour aliquots analyzed.

Urinary estrogen metabolites were analyzed by the gas chromatographic/mass spectrometric (GC/MS) methods of Fotsis and Adlercreutz[18], although modified by Kurzer and colleagues[19]. These assays were performed by GC/MS analysis on a Hewlett Packard 5971A quadrupole instrument, using selective ion monitoring, after multiple extractions and chromatographic separations. Deuterated internal standards for all estrogen metabolites were used. The two urinary estrogen metabolites used in the analysis include the estrone (E1) and estradiol (E2).

pQCT Measures

Bone Measurements

To obtain measures of bone density and geometry, slices (2.3 ± 0.2 mm) of the left tibia and were obtained at the 4, 50, and 66% sites of the tibia and the 4 and 50% sites of the non-dominant radius proximal to the distal plafond of the tibia and radius using peripheral quantitative computed tomography (pQCT, Norland/Stratec XCT 3000 bone scanner, Stratec Medizintechnic GmbH, Pforzheim, Germany). A voxel size of 0.4 mm was used and the scan speed was set at 25 mm/s. The anatomic reference line was determined by acquisition of a 30 mm planar scout view of the joint line. The distal sites of the tibia and radius were assessed for trabecular volumetric density (TrabDen, mg/mm^3), total volumetric density (ToD, mg/mm^3), and total bone cross-sectional area (ToA, mm^2) using Contour mode 3 (200 mg/cm^3), Peel mode 5 (automatic), and Cort mode 3 (169 mg/cm^3). An estimate of the bone strength (Bone Strength Index, BSI) was calculated as ToA * ToD^2 [28, 29]. The proximal sites were assessed for total bone area (ToA, mm^2), total cortical area (CoA, mm^2), cortical volumetric density (CoD, mg/mm^3), and polar strength-strain index (SSIp, mm^3) using Contour mode 1 (710 mg/cm^3), Peel mode 2 (540 mg/cm^3), and Cort mode 1 (480 mg/cm^3). Threshold values and cort/peel modes were set according to manufactures recommendations. Measurements were made by one of three trained operators and a single operator will analyze all scans. A pQCT anthropomorphic phantom was scanned daily to maintain quality assurance.

Body Composition

Body composition was measured by dual energy x-ray absorptiometry (DXA, Lunar Prodigy, Lunar Corp., Madison, WI). Total body scans were taken at the University of Minnesota's General Clinical Research Center (GCRC) and analyzed using manufacturer software (Lunar v 5.54) for total body lean and fat mass. All measurements were taken by certified technicians. Previous research has shown CV for DXA measured fat mass and lean mass to be 1.9% and 1.5% respectively[20]. A digital scale and a scale mounted stadiometer (Scale-tronix 5005 stand-on digital scale, Scale-tronix, White Plains, NY), calibrated weekly, was used to measure weight (to the nearest 0.1 kg) and height (to the nearest 0.1 cm).

Statistical Data Analysis

All statistical analyses were conducted using SPSS software (version 13.0; SPSS Inc., Chicago, IL). Scatterplots were used to identify outliers and any points that were at least 3.0 SD away from the group mean were excluded (n=3). Histograms were created to check for the normality of the data. Descriptive characteristics are reported as means and standard deviations (SD). Participants were categorized into tertiles of lean mass and estrone levels. We used analyses of variance (ANOVA) in to test for intergroup differences in age, height, weight, calcium, and menstrual cycle characteristics. To test for interactions between urinary estrogen levels, total body lean mass, and bone strength, we used an ANCOVA to determine overall significance. The base model was adjusted for age, weight and height.

Results

Descriptive characteristics (mean$\pm$SD) are reported of the population (Table 1). To examine the relationships among lean mass, estrogen, and bone strength and geometry, we compared geometric properties among subjects divided into tertiles of lean mass (LM) and of estrone (E1). There exists a possibility that LM or E1 tertiles might differ in other variables that influence bone strength, so we first tested for differences in age, height, limb length, weight, calcium and menstrual cycle characteristics (Table 2). Comparing estrone tertiles, there were significant differences in levels of estrone ($p<0.05$). Comparing LM tertiles, the women with the greatest amount of lean mass were taller, heavier and had greater levels of LM ($p<0.001$).

Table 5-1. Descrpitive characteristics of sedentary women aged 18-30

	N	Mean (SD)
Age (years)	64	25.66 (3.11)
Height (cm)	64	164.94 (7.95)
Weight (kg)	64	65.18 (14.32)
BMI	64	23.89 (4.52)
Body fat (%)	64	34.81 (7.79)
Lean (kg)	64	39.31 (5.37)
LPL (days)[a]	61	13.18 (2.11)
MCL (days)	58	29.95 (4.90)
Calc (mg)	52	911.80 (458.25)
Estradiol (ng/mL)[b]	64	61.56 (19.21)
Estrone-S (ng/mL)[b]	64	2.50 (0.86)

[a]Luteal phase length is based on time between ovulation and onset of menses
[b]Urinary estrogen measures are averaged from days 7-9 of menstural cycle

Table 5-2. Age, height, weight, estrone (E1), lean mass (LM), calcium, luteal phase length (LPL) and menstrual cycle length (MCL) by E1 and LM tertiles.

		LM Tertiles			**E1 Tertiles**		
	N	LM 1	LM 2	LM 3	E1 1	E1 2	E1 3
Age (yr)	64	25.62 (3.47)	26.18 (2.40)	25.14 (3.44)	26.24 (2.84)	25.55 (2.82)	25.19 (3.67)
Height (cm)	64	**159.03 (4.50)[a]**	163.04 (5.92)	**172.84 (5.97)[a]**	165.10 (8.88)	164.92 (7.43)	164.80 (7.88)
Weight (kg)	64	**54.35 (6.67)[a]**	65.02 (8.69)	**76.17 (16.51)[a]**	68.4 (17.82)	63.21 (10.03)	64.01 (14.37)
Calcium (mg)	52	1006.23 (561.39)	758.43 (392.96)	953.71 (380.96)	785.52 (337.90)	902.23 (324.25)	1026.72 (614.96)
Lean mass (kg)	64	**33.61 (2.75)[a,c]**	**39.15 (1.39)[a,b]**	**45.16 (3.35)[b,c]**	39.44 (6.22)	38.93 (4.32)	39.57 (5.70)
LPL (d)	61	13.5 (2.38)	12.9 (2.12)	13.15 (1.86)	13.13 (1.92)	13.19 (2.22)	13.23 (2.29)
MCL (d)	58	29.82 (3.69)	31.6 (6.92)	28.34 (2.48)	30.61 (7.36)	29.71 (3.21)	29.55 (3.19)
E1 (ng/ml)	64	2.43 (0.66)	2.47 (0.96)	2.61 (0.96)	**1.73 (0.30)[a]**	**2.47 (0.96)[b]**	**3.41 (0.80)[a,b]**

Reported as mean ± SD
[a,b,c] $p<0.05$, ANOVA

Lean Mass

We tested the effects of LM on bone geometry and strength (Table 3). As predicted, lean mass is significantly associated with young adult bone geometry and bone strength as measured by SSIp. Those young women who had the greater amounts of LM had higher SSIp and cortical area at the 66% tibia ($p<0.01$ for groups 2-3 versus group 1) and greater 66% total area ($p<0.05$, group 2 vs. 1). At the 50% site, women in the higher LM tertiles had greater cortical and total area at the 50% tibia ($p<0.01$ for groups 2-3 vs. group 1) compared to women with the lowest amount of lean mass. There was also a significant effect of LM on vBMD at the 4% tibia ($p<0.05$ for groups 2-3 vs. group 1).

Table 5-3. Bone density, geometry and estimates of strength for tertiles of lean mass (LM) in sedentary young adult women.

	Tertile of Lean Mass		
	Tertile 1	Tertile 2	Tertile 3
4% Tibia			
ToA (mm^2)	904.70 (694.53, 1114.87)	904.25 (747.09, 1061.41)	899.79 (691.56, 1108.03)
ToD (mm^3)	283.10[a,b] (176.61, 389.59)	322.14[a] (242.51, 401.77)	341.09[b] (235.58, 446.60)
BSI (mg^2/mm^4)*	237.06 (171.61, 302.51)	262.55 (213.60, 311.49)	276.31 (211.46, 341.16)
50% Tibia			
ToA	365.8[a,b] (293.16, 438.44)	411.51[a] (354.78)	414.75[b] (336.54, 492.96)
CoA	258.71[a,b] (200.94, 316.48)	291.62[a] (246.51, 336.73)	299.39[b] (237.19, 361.59)
CoD	1185.42 (1129.77, 1241.07)	1173.62 (1130.16, 1217.08)	1176.97 (1117.05, 1236.89)
SSIp	1648 (1290.12, 2007.16)	1641.08 (1363.38, 1918.77)	1803.54 (1419.53, 2187.54)
66% Tibia			
ToA	487.05[a] (372.09, 602.02)	527.22[a] (438.79, 615.65)	533.01 (411.53, 654.48)
CoA	250.43[a,b] (184.87, 315.99)	289.07[a] (238.64, 339.50)	298.94[b] (229.67, 368.21)
CoD	1158.16 (1103.08, 1213.27)	1145.14 (1102.76, 1187.53)	1140.41 (1082.19, 1198.64)

SSIp	1959.42[a,b] (1596.31, 2322.53)	2286.09[a] (2006.78, 2565.40)	2366.12[b] (1982.45, 2749.79)

Values are Mean (95% confidence intervals)
[a,b]p<0.05, ANCOVA, adjusted for age, height and weight

Estrone

We also compared bone geometry and strength values among estrone (E1) tertiles (Table 4). At the 66% site, we found that women in the highest tertile had significantly greater SSIp ($p<0.05$ for group 3 versus groups 1-2), cortical area ($p<0.05$ for group 3 versis group 2), and total area for cortical and total area ($p<0.05$ for group 3 versus group 2). At the 50% site, women in the highest tertile also had significantly greater cortical and total area ($p<0.05$ for group 3 versus group 2).

Table 5-4. Bone density, geometry and estimates of strength for tertiles of estone (E1) in sedentary young adult women.

	Tertile of Estrone		
	Tertile 1	Tertile 2	Tertile 3
4% Tibia			
ToA (mm^2)	904.88 (756.60, 1053.16)	907.61 (741.49, 1073.72)	896.26 (740.70, 1051.83)
ToD (mm^3)	326.30 (246.36, 406.23)	309.33 (219.77, 398.88)	314.65 (230.78, 398.52)
BSI (mg^2/mm^4)*	268.87 (221.58, 316.17)	253.74 (200.75, 306.72)	255.46 (205.84, 305.08)
50% Tibia			
ToA	396.22 (334.23, 458.20)	386.36[a] (326.45, 446.27)	410.64[a] (347.64, 473.64)
CoA (mm^2)	285.41 (236.70, 334.12)	273.63[a] (226.55, 320.70)	291.55[a] (242.04, 341.05)
CoD (mm^3)	1181.78 (1137.91, 1225.66)	1177.55 (1135.15, 1219.95)	1176.63 (1132.04, 1221.22)
SSIp (mm^3)	1699.07 (1412.34, 1985.80)	1654.13 (1383.98, 1924.28)	1742.19 (1465.42, 2018.96)
66% Tibia			
ToA	500.23[a] (411.98, 588.48)	508.57 (424.81, 592.33)	540.63[a] (452.87, 628.38)
CoA	282.10 (226.20, 338.01)	268.85[a] (215.79, 321.91)	290.00[a] (234.41, 345.59)
CoD	1153.69 (1110.35, 1197.04)	1145.20 (1104.06, 1186.34)	1144.59 (1101.48, 1187.69)
SSIp	2155.38[a] (1864.68, 2446.04)	2113.13[b] (1837.25, 2389.01)	2364.43[a,b] (2075.39, 2653.47)

Values are Mean (95% confidence intervals)

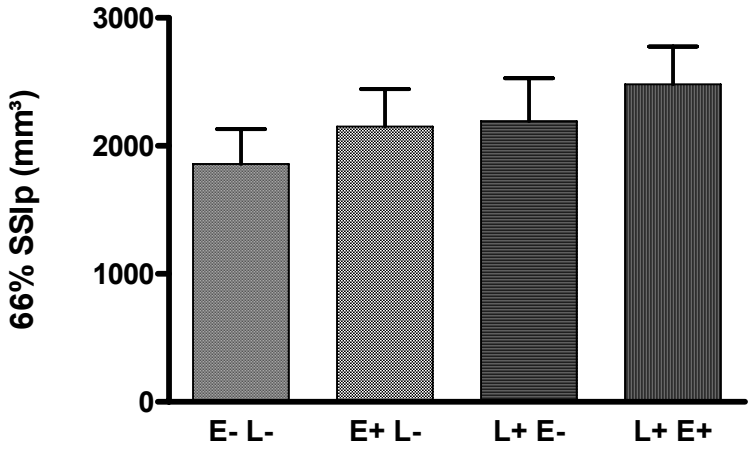
[a,b]$p<0.05$, ANCOVA, adjusted for age, height and weight

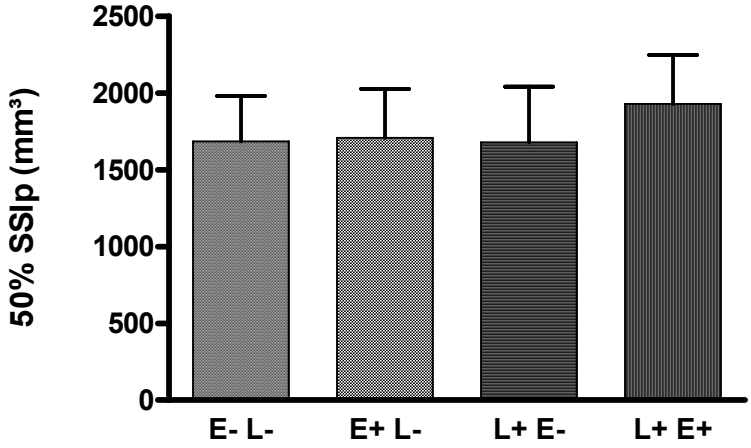
Combined Effects

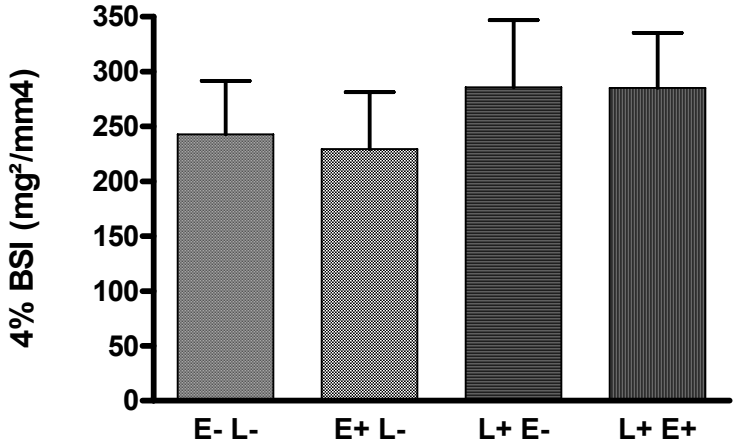
Although lean mass and estrone levels are each independently associated with young adult bone strength at the 66% site, we found no statistical support for and interaction effect of E1

and LM on bone strength (Figure 1). However, we observed that at the 66% site, women in the highest LM and E1 tertiles (N=6) have 33% higher SSIp than do women in the lowest PA and E2 tertiles (N=6), and a 14.4% higher SSIp at the 50% site (N=6 in the lowest tertiles group, N=8 in the highest tertile groups). Given that E1 and LM are each independent predictors of bone strength, it seems likely that a larger sample size might reveal significant E1*LM interactions.

Figure 5-1. Interaction of estrone (E1) and lean mass (LM) on bone strength







Discussion

Our data support the hypothesis that urinary estrone levels and lean mass have positive significant independent effects on bone strength. We found that participants who had the greatest amount of lean mass had 20**%** greater bone strength at the tibial midshaft. Both cross-sectional and intervention studies have concluded that various forms of mechanical loading, through physical activity and athletic participation, have beneficial effects on bone density in premenopausal women[21-24], and measures of lean mass are a strong surrogates of the mechanical load on the bone[6, 25]. Similarly, women who had the highest estrone levels

had 10**%** greater bone strength at the 66% tibia site, compared to women with lower estrone levels ($p<0.05$). We also found significant independent effect of estrone levels on total area at the 66% tibia. This finding expands the role of estrogen to possibly include effects on bone geometry, which would lead to stronger bones.

In contrast to our hypothesis, we did not observe a significant interaction between estrogen and lean mass in their effects on bone strength. Previous studies showed both a significant interaction[26-28], and no interaction[29, 30]. Our findings are in accordance with the recent finding by Pajamaki and colleagues, who found that there was no significant interaction between estrogen and loading on bone geometry and strength[30]. Estrogen is thought to influence bone adaptation mechanical loads by lowering the theoretical mechanostat setpoint on the endosteal surface[1, 12, 31]. As a result, exercise may have a more beneficial effect on endosteal bone formation in the presence rather than the absence of estrogen. However, estrogen does not appear to be anabolic on periosteal bone. Research suggests that estrogen-replete women may be less sensitive to loading-induced periosteal bone formation compared to prepubertal girls[32, 33]. Much of currently published clinical studies of estrogen and loading interactions lack reliable bone mass and strength data[34-36]. Consequently, there is a great need to further investigate these issues in this population using peripheral quantitative tomography (pQCT) in vivo to derive bone strength indices. Lack of research looking at the relationship between loading and sex steroid levels leaves many unanswered questions as to the true effect of estrogen on bone "health" in healthy premenopausal women, especially using pQCT or other technology that can give a more accurate description of the architecture and geometric properties of bone.

These results expand upon previous studies by demonstrating significant independent effects of estrogen and lean mass on measures of bone geometry and strength, as measured by pQCT, in young adult women[30, 37-39]. Also, this study suggests that there is no interaction between estrone and LM, However The fact that the women in the study were all healthy and eumenorrheic, the differences in estrone levels may not have been great enough to determine an interaction effect on bone measures. Thus estrogen does not appear to increase the osteogenic response to mechanical loading in healthy, eumenorrheic young women. Studies that compare women with menstrual cycle disturbances, such as amenorrhea or olgimenorrhea, to a healthy eumenorrheic population may be needed to possibly determine the role of estrone or other estrogen metabolites on bone adaptation to mechanical loads.

Given the cross-sectional nature of this study, we cannot determine a causal relationship between estrone, LM and bone strength, and hence longitudinal data is needed to confirm the relationship among these factors. Also, the one time measure estrone does not account for monthly variation in estrone. Nonetheless, this study provides evidence of a possible relationship between estrone and bone strength and between LM and bone strength in this population. Future research is needed to support these findings.

In conclusion, this study demonstrates that young women's estrone levels and LM have important consequences for the development of young adult cross-sectional geometry and therefore, of bone strength. Lean mass is a well-known modifiable determinant of bone

strength, through increasing physical activity, but estrogen bioavailability may not be modifiable. If low estrogen and LM levels reduce bone strength, specific populations may at risk for skeletal fragility, such as young women with amenorrhea, anorexia, or hypogonadism[40-42], and in sedentary women. Future studies are required to further clarify the relationships among estrogen, mechanical loading, and bone growth and adaptation in young women.

6

OVERALL SUMMARY AND CONCLUSIONS

6. SUMMARY AND CONCLUSION

6.1 Bone Strength and Geometry in Premenopausal Women

In Parts I and II, I present results from the most comprehensive cross-sectional pQCT studies of sedentary young adult women conducted to date. These data add to the current body of knowledge regarding predictors of bone strength and geometry, and the independent effects of estrogen and lean mass on these bone outcomes. These studies also highlight the need to measure estrogen and menstrual cycle characteristics, in this population. Women in this age range (18-30) are often overlooked in bone research, but my data suggests that young women may be at an increased risk for skeletal fragility if they have low levels of lean mass and estrogen, along with poor nutrition. In addition, this study provides the first pQCT human evidence of: 1) the relationship between urinary measures of estrogen and measures of bone geometry and strength and 2) the effects of subtle changes in menstrual cycle characteristics on bone radial vBMD and tibial area and strength. The WISER study has provided me with hormonal measures and characteristics not found in any other pQCT study of this population to date, along with access to young eumenorrheic women not on birth control, a difficult population to recruit.

My data also provides absolute pQCT bone values, which are lacking in the current literature. There are currently no reference data for this population, which are vital to future applications of pQCT outcomes in all populations. As such, there is an immediate need for standardization of pQCT acquisition and analysis protocols for the assessment of young adult women bone, to include the reference line, resolution (voxel size), measurement sites, analysis modes, thresholds and outcome variables. Defining these factors will allow for comparisons of results across studies and help define reference data.

Given the cross-sectional nature of these studies, there is a need to explore these relationships further in longitudinal studies of changes in pQCT measures over time in conjunction with changes in body composition, hormonal levels, and menstrual cycle characteristics. Also, there is a need to explore the effect of a weight-bearing mechanical loading intervention of 12-16 months on bone strength, since it is thought that it takes that long for bone to fully mineralize and be detectable by bone measurement devices. With

appropriate longitudinal data, we will be able to get further insight into the functional model and of bone adaptation along with providing support the theoretical framework (mechanostat).

Nonetheless, my study results suggest that young adult otherwise healthy women may be at an unknown increased risk for skeletal fragility because of low levels of lean mass and/or estrogen. This study has provided data that encourages much further research of bone health in this population.

6.1.1 Part I: Predictors of Bone Strength and Geometry in Young Premenopausal Women

Summary *(Primary Objectives):*

After adjusting for height and age:

a) Tibial and radial bone strength indices (BSI, SSIp) were primarily predicted by surrogate measures of mechanical load (lean mass, muscle cross-sectional area (MCSA)). Lean mass predicted 20% of proximal tibial SSIp and 16% of distal tibial BSI. At the proximal radius, MCSA predicted 47% of SSIp.
b) Volumetric bone mineral density (vBMD) at the tibia (loaded bone) was primarily predicted by body composition (fat mass of lean mass). Lean mass predicted 8% of vBMD at the distal tibia, and fat mass predicted 15-20% of vBMD at the proximal tibia.

Summary *(Secondary Objective)*

After adjusting for height and age:

a) Menstrual cycle characteristics primarily predicted radial (non-loaded bone) vBMD with menstrual cycle length (MCL) predicting 5% of distal vBMD and luteal phase length (LPL) predicting 6% of proximal vBMD. MCL also significantly contributed to proximal tibia area and SSIp.
b) A measure of estrogen (E1) explained 3-5% of proximal tibia bone area.

c) Nutrition explained only a small amount of the variance of bone. outcomes. Protein explained 4% of proximal radial area, while vitamin D explained 6% of distal tibial area.

Conclusions:

a) In the accordance with the mechanostat, surrogates of mechanical load on bone are associated with beneficial adaptation of bone structure and strength in sedentary young premenopausal women.
b) Estrogen is a significant predictor of tibial bone area and strength in the same population.
c) Subtle changes in menstrual cycle characteristics in eumenorrheic women may effect vBMD geometry, resulting in significant changes in bone strength.
d) In young women, fat mass may be significantly inversely associated with tibial vBMD, but only lean mass appears to effect overall bone strength.
e) Nutrition plays only a minor role in bone geometry, but does not seem to effect bone strength.
f) Changes in lifestyle factors (physical activity and nutrition) may increase bone strength during the premenopausal years.

6.1.2. Part II: Independent and interactive effects of lean mass and estrogen on bone strength and geometry in sedentary young adult women.

Summary *(Primary Objective):*

After adjusting for age, height and weight:

a) Women with the greatest amount of lean mass had 20% greater bone strength at the proximal tibia.
b) Women with the highest levels of estrogen had 10% greater bone strength at the proximal tibia.
c) Estrogen and lean mass were independently associated with measures of vBMD at the proximal tibia. Women with the highest level of estrogen had 6.5-8%

greater cortical area, and those with the highest level of lean mass had 16-19% greater cortical area.

Summary *(Secondary Objective):*

d) No significant interaction was found between estrogen and lean mass on bone geometry and strength, but women in the highest tertile of both lean mass and estrogen had 33% greater SSIp compared to women in the lowest tertiles.

Conclusions:

a) Lean mass and estrogen appear to have significant effects on measures of proximal tibia strength and geometry.
b) Lean mass was more significantly associated with measures of strength and geometry than was estrogen.
c) The trend for women with the greatest levels of lean mass and estrogen suggests that women with diseases or disorders that cause below average circulating estrogen, such as anorexia or amenorrhea, may be at risk for skeletal fragility.
d) Increasing lean mass, through physical activity, may increase bone strength in sedentary women.

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7

APPENDICES

A. CONSENT FORM

Consent Form

Women, Oxidative Stress, Estrogens, and Exercise: Bone and Muscle Strength in Pre-Menopausal Women

Moira Petit PhD, Beth Kaufman, Amanda Thieschafer MA, Mindy Kurzer PhD
University of Minnesota

You are invited to participate in a research study to explore the role of hormones on bone and muscle strength. You were selected as a possible participant because you meet the study's qualifying criteria for inclusion in the study. We ask that you read this form and ask any questions you may have before agreeing to be in the study.

1 Study Purpose

The purpose of the study is to use a new bone imaging device to determine bone and muscle strength in healthy pre-menopausal females with regular menstrual cycles. We anticipate these data will provide insight into the role of hormones on bone and muscle development.

2 Study Procedures

If you agree to participate in this study, we would ask you to come to the University of Minnesota's Laboratory for Musculoskeletal Health (LMH) housed in the University Recreation Center for one visit approximately 30 minutes long. We will measure your weight using a scale and measure your height, sitting height, leg length, and forearm length using a tape measure. These measurements will take approximately 10 minutes

After we take these measurements, we will measure your bone density, bone geometry (shape) and amount of muscle in your lower leg and forearm using a peripheral quantitative computed tomography (pQCT). This is a CT scan with a low dose of radiation to the forearm and lower leg. You will be asked to sit with one leg extended and secured within the pQCT for 15 minutes for measurements of the leg followed by measurements of the forearm, in which you will be asked to sit with one arm extended and secured within the pQCT for 5 minutes. We will use the pQCT to take 3 scans of your lower leg and 2 scans of your forearm.

3 Risks of Study Participation

As part of this study you will undergo 1 peripheral quantitative computed tomography (pQCT) procedure. This procedure involves exposure to ionizing radiation. The amount of ionizing radiation you will receive from this procedure is less than 1% of that received each year by a Minnesota resident from natural background radiation (300 mrem/yr).

Participants will be asked if they are pregnant or are planning on becoming pregnant, and those who are pregnant, or plan to become pregnant will not be included in the study. If you are unsure of their pregnancy status, let the researcher know, and a pregnancy test will be administered prior to any pQCT measurements. Participants under the age of 18 cannot participate in this study. Also, if you have participated in any other study in the past 12 months, please inform the investigator for documentation of possible previous exposure to radiation.

4 Benefits of Study Participation

This study has no direct benefits for the participant.

5 Study Costs/Compensation

THERE ARE NO COSTS TO THE PARTICIPANT TO BE A PART OF THE STUDY.

6 Research Related Injury

In the event that this research activity results in an injury, treatment will be available, including first aid, emergency treatment and follow-up care as needed. Care for such injuries will be billed in the ordinary manner to you or your insurance company. If you think that you have suffered a research related injury, let the study physicians know right away.

Confidentiality

The records of this study will be kept private. In any publications or presentations, we will not include any information that will make it possible to identify you as a subject.

7 Voluntary Nature of the Study

Participation in this study is voluntary. Your decision whether or not to participate in this study will not affect your current or future relations with the University. If you decide to participate, you are free to withdraw at any time without affecting those relationships.

Contacts and Questions

You may ask any questions you have now, or if you have questions later, you are encouraged to contact Dr. Moira Petit at 612-625-5506 (email: mpetit@umn.edu) or Beth Kaufman at 612-625-9753 (email: moen0177@umn.edu).

If you have any questions or concerns regarding the study and would like to talk to someone other than the researcher(s), you are encouraged to contact the Fairview Research Helpline at telephone number 612-672-7692 or toll free at 866-508-6961. You may also contact this office in writing or in person at University of Minnesota Medical Center, Fairview-Riverside Campus, #815 Professional Building, 2450 Riverside Avenue, Minneapolis, MN 55454.

You will be given a copy of this form to keep for your records.

Statement of Consent

I have read the above information. I have asked questions and have received answers. I consent to participate in the study.

Signature of Participant_____
Date_____

Signature of Investigator_____
Date_____

University of Minnesota

Become a WISER Woman

www.wiserwomen.umn.edu

Name: _____

January 2008

Sun	Mon	Tue	Wed	Thu	Fri	Sat
		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	31		

Additional Notes/Comments:

Remember to Note

- Start of menstruation, time of day, cycle number, and flow perception (*PI/AM/Moderate/Anxiety, Cramps). Where period flow is categorized as:
  - Very heavy, heavy, moderate, somewhat light, or very light

  And symptoms include:
  - Acne, anxiety, backache, bloating, breast swelling, breast tenderness, cramps, fatigue, food craving, headache, irritability, joint pain, moody, muscle pain/aches, nausea, tension, water retention, or weight gain
- Urine collection dates (urine)
- Food record dates (fd rec)
- Scheduled clinic visits with GCRC (CV1)
- Urine pick-up (PU)
- Ovulation test results (-OVU or+ OVU)
- Last day of menstrual flow/period (**PI)
- Number of days in menstrual cycle (+1, +2, … +28)

wiser