Use of Micro-Computed Tomography to Evaluate the Changes to Bone Structure due to Ovariectomy and Polyunsaturated Fatty Acid Intervention in a Rat Model of Postmenopausal Osteoporosis

Amanda B. Longo, M.Sc.

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Faculty of Applied Health Sciences
Brock University
St. Catharines, ON.

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Abstract

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The testing of osteoporosis-related therapies and the investigation into their mechanism of action largely relies on the use of the preclinical model of postmenopausal osteoporosis, the ovariectomized (OVX) rat. Study 1 evaluated the time-course of changes to the trabecular and cortical microarchitecture of the proximal tibia of the Sham-control and OVX rat over a 3-month period following surgery at 3-months of age, a typical period used in the testing of nutritional and/or drug intervention. This study confirmed the use of the 3-month OVX rat as a model for trabecular microarchitecture changes, but did not support its use as a model for cortical bone changes. The OVX rat was also used to test its resistance to repeated radiation exposure from micro-computed tomography (µCT), a method necessary for the longitudinal evaluation of a single animal (Study 2). The method of µCT and its scanning parameters were also investigated to confirm their use in appropriately quantifying the 3-dimensional microarchitecture of the rat hind limb both *in vivo* and *ex vivo* (Study 3). Together, findings from these methodological studies led to its use in the evaluation of changes to bone structure in response to a nutritional intervention in the OVX rat (Study 4). Previous studies of growing rats and of OVX rats suggest a benefit to bone with supplementation with a
lower n-6 to n-3 polyunsaturated fatty acid (PUFA) dietary ratio, and to altered sources of n-3 PUFA, but no study has investigated the impact of these nutritional patterns over the life course. Therefore, Study 4 determined the effect of the dietary ratio and source of n-3 PUFA on bone during periods of growth (1 through 3 months of age) and following Sham or OVX surgery (at 3 months of age). A low PUFA ratio with n-3 provided from flaxseed oil provided benefits to bone health during growth, but these changes were not maintained following OVX. These data suggest that dietary PUFA, at levels attainable in humans through a healthful diet, are not an adequate strategy for the prevention of the rapid and drastic bone loss induced by OVX.
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List of Abbreviations

AA: arachidonic acid
ALA: alpha-linolenic acid
BEST: The Bone Estrogen Strength Training Study
BMC: bone mineral content
BMD: bone mineral density
BV/TV: percent bone volume
Conn.D: connectivity density
COX: cyclooxygenase
Ct.Ar.: cortical area
Ct.Ar./Tt.Ar.: cortical area fraction
Ct.Th.: cortical thickness
CTX: C-terminal telopeptide
DA: degree of anisotropy
DHA: docosahexaenoic acid
DPA: docosapentaenoic acid
DXA: dual energy X-ray absorptiometry
Ec.Pm.: endocortical perimeter
Ecc: eccentricity
EPA: eicosapentaenoic acid
FRAX: fracture risk assessment tool
LA: linoleic acid
LOX: lipooxygenase
LV: lumbar vertebrae
Ma.Ar.: medullary area
MUFA: monounsaturated fatty acid
NHANES: National Health and Nutrition Examination Survey
NHS: Nurses’ Health Study
NTX: N-terminal telopeptide
Obj.V/TV: object area fraction
OPG: osteoprotegerin
OVX: ovariectomy
PGE2: prostaglandin E2
PPAR: peroxisome-proliferator activator receptor
pQCT: peripheral quantitative computed tomography
Ps.Pm.: periosteal perimeter
PUFA: polyunsaturated fatty acid
RANKL: receptor activated of nuclear factor kappa beta ligand
RCT: randomized controlled trial
ROI: region of interest
SFA: saturated fatty acid
SD: Sprague-Dawley
Tb.N.: trabecular number
Tb.Th.: trabecular thickness
Tb.Sp.: trabecular separation
TNFα: tumor necrosis factor alpha
Tt.Ar.: total bone area
WHI: Women’s Health Initiative
µCT: micro-computed tomography
VOI: volume of interest
Contributions

Amanda Longo provided significant intellectual contributions to the study designs with Dr. Ward, was responsible for conducting all of the in vivo and ex vivo experiments, performing all of the analyses, and daily tasks. Amanda was the principal surgeon for the Sham and OVX surgeries performed as a part of Study 4. Amanda performed all of the statistical analyses, interpreted the results, wrote the first draft of each manuscript, and incorporated all feedback from other coauthors. In addition to Dr. Ward, the following list of people contributed to the body of work included in this thesis.

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- Provided expertise and advice regarding the µCT scanner (Study 1 to 4)
- Contributed to the interpretation of data (Study 1 to 3)

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- Performed analysis of radiation dose experiments (Study 2)
- Contributed to the interpretation of data (Study 1 to 3)

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- Contributed to the study design and interpretation of data (Study 4)

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- Contributed to the design and interpretation of statistical analyses (Study 4)

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- Performed a subset of µCT scans, reconstruction, and analysis (Study 3)

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- Provided insight into the high standard of animal care and was a driving force for the continued advancement of ethical procedure and policy (Study 1 to 4)
Chapter 1: Introduction
Introduction

Osteoporosis is a disease of the skeleton, characterized by a decline in bone mass (bone quantity) and a deterioration of the bone tissue (bone quality) (1). Together, the decline in bone quantity and quality lead to an increased risk of sustaining a fragility fracture, often resulting in substantial morbidity and a reduced overall quality of life. Women are disproportionately affected by osteoporosis, with 1 in 3 women and only 1 in 5 men suffering from an osteoporotic fracture in their lifetime (1). This imbalance in fragility fracture incidence is due to the loss of endogenous estrogen, experienced by women following menopause, causing an imbalance in bone turnover, favouring bone resorption. One potential strategy for reducing the incidence of experiencing a fragility fracture later in life is by increasing the peak bone mass acquired during periods of growth and development in early life. Although approximately 20% of peak bone mass is determined by genetic factors (2), other strategies including exercise and healthful nutrition can increase peak bone mass. This ultimately leaves more bone mass (quantity) available to be resorbed during the menopausal transition before a high risk of fragility fracture is reached.

One potential strategy for increasing bone mass (quantity) and bone structure (quality) during growth and for preserving bone mass and structure following the loss of endogenous hormone production is with the incorporation of PUFA in the diet. The n-6 and n-3 series of PUFA, and their ratio within the diet, have been shown to influence bone cell metabolism, and ultimately alter bone health-related outcomes. Therefore, a main objective of this thesis (Study 4, Chapter 7) was to determine how the ratio of n-6 to n-3 PUFA in the diet, at levels attainable through healthful dietary patterns, affect bone
quantity and bone quality throughout periods of growth, and following the cessation of estrogen production. The source of n-3 provided from plant or marine oil sources was also tested. Findings from Study 4 showed that providing diets with a lower n-6 to n-3 PUFA diet with n-3 provided from flaxseed oil resulted in changes to bone health during periods of growth in a pre-clinical model. These results suggest that bone health can be altered by a dietary intervention during periods of life predominated by bone modeling. The effects of these diets are not maintained following the removal of the ovaries in the preclinical model, suggesting that diet is not an adequate strategy for protecting against hormone-related bone loss in females.

The use of the OVX rat model as a preclinical model for postmenopausal osteoporosis has been well characterized and has long been used in the literature for the assessment and development of osteoporosis-related treatments. However, with the advent of in vivo µCT, a method that allows for the repeated assessment of bone quantity and 3-dimensional bone quality (trabecular and cortical microarchitecture), the changes to bone microarchitecture in the OVX rat model must be further elucidated. Therefore, a primary objective of this thesis (Study 2-3, Chapter 5 and 6) was to characterize a reliable method for the repeated in vivo quantification of trabecular and cortical bone of the proximal tibia of the rat. These methods were used to determine the time-course of trabecular and cortical microarchitecture changes to the proximal tibia of Sham-operated and OVX rats (Study 1, Chapter 5). Results from Study 1 led to the recommendation for the use of the 3-month OVX Sprague-Dawley (SD) rat as a model for trabecular bone loss. The proximal tibia of this model should not however, be used for the assessment of changes to cortical bone.
The overall experimental approach of this thesis provided a comprehensive understanding of the method of *in vivo* µCT scanning, the characterization of the trabecular and cortical microarchitecture outcomes of the 3-month OVX rat model of postmenopausal osteoporosis, and the effect of exposure to a nutritional PUFA intervention over the life course. The primary research presented in this thesis provides the basis for the experimental design of future studies utilizing µCT and the 3-month OVX rat model and provides a foundation for future studies to be conducted using male rats and other aged rodent models. This thesis also provides an experimental basis for future studies aimed at assessing the effects of PUFA ratio and n-3 source on the attainment of peak bone mass in humans, during adulthood, throughout the menopausal transition and following menopause, and throughout the entire lifespan.
Chapter 2: Literature Review

Modified in part from (3):

The Skeleton

The skeleton is a specialized supporting framework of the body which provides shape and support, protection for vital organs, facilitates the production of hematopoietic marrow, and acts as a mineral reserve for calcium and phosphorus homeostasis (4).

Bone Structure and Function

Bone is made up of a series of woven type I collagen fibers that is stiffened by hydroxyapatite molecules (made from calcium and inorganic phosphate) and undergoes constant remodeling to obtain an optimal mass, shape, and architecture (5). The calcium and phosphorous in hydroxyapatite molecules in bone can be mobilized to maintain mineral homeostasis in the body when needed for other functions (4). As well as acting as a storage unit for minerals, the skeleton is a metabolically active organ constantly undergoing the process of remodeling to adapt to the mechanical loads being applied and to maintain its structural integrity, all the while retaining its flexibility and remaining as light as possible to allow for efficient locomotion. While changes to bone mass and structure are dominated by mechanical factors, non-mechanical agents such as nutrition, hormones, and genetics can also influence bone mass (6). Thus, bone is a dynamic tissue, constantly undergoing changes to its shape and architecture at the microscopic level. For this thesis, I focused on the individual and combined effects of nutrition and hormones, specifically PUFA and the presence or absence of estrogen on bone health in the OVX rat model of postmenopausal osteoporosis.

Trabecular and cortical bone are the predominant forms of bone tissue in the skeleton (Figure 2-1). Trabecular bone makes up roughly 20% of the adult skeleton and is primarily found in the epiphysis and metaphysis of the long bones, and in the vertebral
bodies. Trabecular bone consists of a network of trabeculae whose 3-dimensional distribution is directionally controlled by the mechanical strains applied to it (7). Because the surface area to volume ratio is so much greater in trabecular than in cortical bone (trabecular bone surface represents 60% of the total surface), the metabolic rate is nearly 10 times higher in trabecular bone (8). Cortical bone is dense and solid and forms the outer shell of the long bones and is more stiff and rigid than trabecular bone. Cortical bone has an outer periosteal surface and an inner endocortical surface. With aging, resorption typically exceeds formation on the endocortical surface, leading to an increase in bone diameter and an expansion of the marrow cavity (9). Therefore, it was important to differentiate and to assess trabecular and cortical bone separately to fully understand the response of the skeleton to an intervention and to hormone status.
Figure 2-1. Structure of the trabecular and cortical bone of the rat tibia

Representative image from a 6-month SD rat having undergone Sham surgery at 3 months of age. Trabecular bone is prevalent in the epiphysis and metaphysis of the long bones, which are compartments separated by the epiphyseal growth plate. The shaft of the long bones contains a high proportion of cortical bone that surrounds the medullary (marrow) cavity and trabecular bone. The surfaces of the inner and outer cortical bone are the endocortical and peristeal surfaces, respectively.

(Image by Longo AB, unpublished, 2016).
Bone Cells

Osteoblasts are mononucleated cells, derived from mesenchymal stem cells, and are responsible for the secretion and deposition of bone matrix for the process of ossification (4). The major product of osteoblasts is type 1 collagen, which is mineralized with hydroxyapatite crystals (10). Osteoclasts are multinucleated cells, derived from hematopoietic stem cells, and resorb bone mineral by secreting protons and the proteolytic enzyme, cathepsin K, through highly active ion channels on the membranes into the extracellular space, ultimately lowering the pH in the environment near the bone surface and dissolving hydroxyapatite (11, 12). Osteocytes are the third major cell type of the bone, which are a series of terminally differentiated osteoblasts that have become trapped in their own secreted bone matrix. Osteocytes are the most abundant cell type in bone and act as mechanosensors (13). These cells extend processes through the surrounding bone matrix to communicate with each other through gap junctions to synchronize bone metabolism (14, 15).

Bone Modeling and Remodeling

The cellular mechanisms responsible for the constant turnover and adaptation of bone to external forces are modeling and remodeling. Bone modeling (formation) occurs at the level of the osteoblast, and involves a change in shape of the bone when new mineral matrix is deposited on surfaces without previous bone resorption (5). Bone remodeling (reconstruction) occurs when new bone is laid down onto surfaces that have been resorbed by osteoclasts. The osteoblast and the osteoclast are the two main cell types of the bone, and together make up the bone multicellular unit that is responsible for the constant turnover of bone in small pockets on its surfaces throughout the life course.
Bone formation and resorption is under strict control by an ever-changing combination of genetic, mechanical, hormonal, and nutritional factors (6). During growth and development, bone formation outweighs resorption to establish peak bone mass, the highest quantity of bone mass present at the end of skeletal maturation. In adulthood, bone resorption to remove damaged bone is balanced with formation to restore the underlying structure, resulting in no net change in bone mass (16). With aging and estrogen deficiency, as seen with menopause, the rate of resorption exceeds the rate of formation on the endocortical surfaces of bone (4). This leads to a decrease in bone mass and compromised bone structure. For this thesis, I have intervened during both the process of modeling and of remodeling. I have introduced a dietary intervention at age 1 month, during bone modeling, with the intervention continued through periods of remodeling when estrogen deficiency was induced by OVX at 3 months of age through to 6 months of age (Study 4).

**Current Methodologies for the Assessment of Bone Health**

There are many current methodologies to assess bone health. These methodologies are ultimately used to identify individuals at risk for sustaining a fragility fracture and to monitor changes to bone health in response to an intervention in both humans and rodents (Table 2-1). Bone health can be assessed by two underlying concepts, bone quantity and bone quality. The assessment of bone mineral content (BMC) and bone mineral density (BMD) provides a measure of the amount of mineral in a defined region. Bone quantity, in the form of BMC and BMD, can be measured with imaging techniques such as dual energy X-ray absorptiometry (DXA), peripheral quantitative computed tomography (pQCT), and µCT. The assessment of the underlying
bone structure provides a measurement of bone quality using imaging techniques such as pQCT (in humans and animals) and µCT (in animals). Computed tomography imaging provides quantification of the microarchitecture of the trabecular and cortical bone. Collectively, bone quantity and bone quality contribute to bone strength and ultimately to its resistance to fragility fracture.
Table 2-1. Strengths and limitations of current methodologies used for the assessment of bone health in humans and rodent models

<table>
<thead>
<tr>
<th>Method</th>
<th>Strengths</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>DXA</td>
<td>• Gold standard for clinical practice and osteoporosis diagnosis • Used to measure BMD at multiple sites • Low radiation dose, high precision, fast measurement time, relatively low cost • <strong>Human and animal use</strong></td>
<td>• Bone size dependent • Does not distinguish between trabecular and cortical bone • BMD does not always predict fracture occurrence</td>
</tr>
<tr>
<td>Risk of Fragility Fracture (FRAX/CAROC)</td>
<td>• Clinically relevant measure • Incorporates multiple risk factors to determine 10-yr fracture risk</td>
<td>• Geographical region-dependent • Online algorithm used • <strong>Human use only</strong></td>
</tr>
<tr>
<td>Bone Derived Biochemical Markers</td>
<td>• Changes suggest either formation or resorption • Respond quickly to an intervention • Can be measured more frequently than imaging methods • <strong>Human and animal use</strong></td>
<td>• High intra-individual variability • Expression is influenced by circadian variation, sampling in a fed or fasted state • Reflect systemic changes; not site-specific</td>
</tr>
<tr>
<td>Biomechanical Strength Testing</td>
<td>• Direct measure of bone strength</td>
<td>• High variability • Limited to <em>ex vivo</em> • <strong>Animal use only</strong></td>
</tr>
<tr>
<td>pQCT</td>
<td>• Quantifies 3D bone structure at higher resolution than DXA • Can differentiate between trabecular and cortical bone • Low radiation dose, high precision • <strong>Human and animal use</strong></td>
<td>• Limited to the axial skeleton in humans • Require specialized scanners and image analysis software</td>
</tr>
<tr>
<td>µCT</td>
<td>• Gold standard for pre-clinical studies of bone trabecular and cortical microarchitecture • Non-destructive method to produce high resolution images <em>in vivo</em></td>
<td>• Exposure to radiation • Requires animals to be anaesthetized • Requires specialized scanners and image analysis software • <strong>Animal use only</strong></td>
</tr>
</tbody>
</table>

1 CAROC; Canadian Association of Radiologists and Osteoporosis Canada, FRAX; fracture risk assessment tool
DXA

BMD as quantified by DXA is currently the gold standard in clinical practice for the diagnosis of osteoporosis by the World Health Organization guidelines (17, 18). The dual energy projection scanning works to quantify BMC in grams by alternative pulses of low current X-ray through the region of interest (ROI) (i.e., hip, wrist, spine, total body), where a detector quantifies X-ray attenuation through the hard and soft tissue and produces an image. BMD is a derived measure and is expressed in g/cm² based on an areal projection. Thus, BMD determined using DXA will differ depending on bone size, and this can cause complications when comparing values across individuals or across studies (19). DXA does not distinguish between trabecular and cortical bone, which can cloud the interpretation of results as changes to areal BMD can be caused by alterations in trabecular bone or from the endocortical surfaces of cortical bone, or both (20).

DXA is advantageous in clinical practice because of its low radiation dose, high precision, fast measurement times, relatively low cost, and ease of use for the technician. BMD can be measured at multiple skeletal sites with ease, including the forearm, hip, lumbar spine, and total body. In one longitudinal study of postmenopausal women, percent changes to BMD over 3 years showed the greatest changes in the spine, followed by the total body and the hip (21), suggesting the lumbar spine may be best when quantifying the response to a treatment in humans. Site-specific BMD measurements show the highest gradients of risk for their respective sites (i.e., forearm BMD best predicts risk of forearm fragility fracture) (22). In fact, measuring changes to BMD using DXA is such common practice that these criteria are recommended for the development and assessment of pharmaceutical interventions in osteoporosis (23). Although the
quantification of BMD by DXA is a well-defined and widespread method, many fragility fractures occur in individuals with T scores above the threshold defined for a diagnosis of osteoporosis (24-26). For this reason, an estimation of fragility fracture risk based on multiple lifestyle factors was developed.

**Risk of Fragility Fracture**

Arguably the most relevant bone health assessment method, the fracture risk assessment tool (FRAX) was developed by the World Health Organization to estimate an individual’s 10-year major osteoporotic and hip fracture risk (27). Using this online algorithm, the probability of fragility fracture can be calculated by combining an individual’s femur neck BMD with known risk factors for osteoporosis: gender, age, body mass index, prior fragility fracture, parental history of hip fracture, current tobacco use, long term use of glucocorticoids (past or present), rheumatoid arthritis, daily consumption of alcohol (>3 servings/day), and other causes of secondary osteoporosis. BMD values are continuously distributed in the population, leaving no guarantee that a fragility fracture will occur at or below a specific threshold. For this reason, the inclusion of several clinical risk factors allow for the more accurate prediction of fracture risk than with BMD alone (22). By incorporating information for the risk factors prior fracture, glucocorticoid use, and a family history of fracture, the 10-year probability of a major osteoporotic fracture for a 65 year old Caucasian women from the United States with a T score of -2.5 increases from roughly 15 to 65% (27). This increase in fracture risk with the inclusion of risk factors highlights the importance of assessing the whole individual, rather than simply the measure of BMD. In addition to the individual clinical risk factors,
fracture probability varies depending on geographical region (28, 29), all of which is taken into account when calculating 10-year fragility fracture risk.

In Canada, the Canadian Association of Radiologists and Osteoporosis Canada risk assessment tool uses criteria similar to FRAX to determine the probability of an individual’s major osteoporotic fracture risk. This assessment method predicts risk of fracture more accurately than with BMD alone (30). From this assessment, a person is classified as having low (<10% 10-year fracture risk), moderate (10-20%), or high risk (>20%) (31). This tool uses BMD, age, gender, prior fragility fracture, and glucocorticoid use and demonstrates high concordance with the Canadian FRAX tool (32).

**Bone Derived Biochemical Markers**

Derived from the cellular and non-cellular components of bone tissue, bone biochemical markers can be quantified in the blood or urine of subjects to determine bone cell activity and bone turnover. These markers are generally classified as either formation or resorption markers and can be measured frequently and relatively non-invasively. Generally, bone formation markers are osteoblast-specific proteins or are from collagen synthesis and bone resorption markers are products of the osteoclast or are a byproduct of collagen breakdown (33).

**The RANKL/RANK/OPG Triad**

The process of bone remodeling, involving the osteoblast and the osteoclast, is regulated in part by the RANKL/RANK/OPG triad (Figure 2-2). Receptor activator of nuclear factor-κβ ligand (RANKL) is a protein secreted by osteoblastic stromal cells, which binds to its receptor RANK, a trans-membrane protein member of the tumor
necrosis factor superfamily (34). Binding of RANKL with its receptor on the surface of osteoclasts and on osteoclast precursors can increase osteoclast activity and induce osteoclast differentiation, respectively (35, 36). Disruption of the RANKL gene in mice results in severe osteopetrosis (abnormally dense bone) and a lack of osteoclasts due to the osteoblasts inability to produce RANKL and drive osteoclastogenesis (37). While ubiquitous overexpression of RANKL in mice is embryonic lethal, overexpression of soluble RANKL expression in the liver after birth results in an increase in osteoclast number with no change in osteoblast number on the surface of trabecular bone (38). These transgenic mice also experience decreases in femur BMD, femur strength, and trabecular bone mass compared to their non-transgenic littermates (38).

Osteoprotegerin (OPG) is a soluble receptor antagonist for RANKL secreted by the osteoblast. OPG functions as a dimer and prevents the binding of RANKL to RANK (39), suggesting its role as an anti-resorptive mediator and RANKL as a promoter of bone resorption during periods of balanced bone metabolism. Studies of transgenic mice deficient in OPG exhibit a decrease in total bone density and an increased incidence of fracture (40), but these outcomes can be reversed with injections of soluble OPG (41). Some studies in humans have even linked polymorphisms in the OPG gene with altered BMD and the risk of osteoporotic fracture (42-44).

OPG and RANKL expression by the osteoblast is tightly regulated by cytokines, hormones, and growth factors (45), and in general, when RANKL expression is up-regulated, OPG expression is down-regulated (36) and vice versa. It has been suggested that OPG concentration should be interpreted as a reflection of overall bone turnover rather than the strict inhibition of resorption because of its increase in serum
concentrations observed with aging and after menopause (46-49). These findings suggest that OPG may provide protection to the skeleton in times of high resorption by inhibiting osteoclast formation, although the relationship between serum OPG concentrations and BMD and fracture risk in postmenopausal women is conflicting (46, 47, 50-52). For this reason, the ratio of serum OPG to serum RANKL is an important factor to consider when examining the balance between bone formation and resorption.
RANKL is a protein secreted from the osteoblast that binds to its receptor, RANK, expressed on the surface of the osteoclast and its precursor cells. RANKL binding to RANK causes the differentiation of and activation of the osteoclast. OPG is a soluble decoy receptor for RANKL, also secreted by the osteoblast. OPG and RANKL secretion by the osteoblast is tightly regulated.

(Image by Longo AB unpublished, 2016. Based on data from (53))
**Biomechanical Strength Testing**

To functionally measure the mechanical integrity of a bone, biomechanical strength testing can be employed. With this method, the strength of a bone can be quantified by determining the amount of compressive stress it can withstand before breaking (54). There are several limitations with this method including: the quantification of the strength of a bone to only one type of stress applied to it (when in nature, multiple stresses and strains are applied at all times) and the high variability associated with the results (55). For these reasons, biomechanical strength testing may be a poor predictor of overall bone strength. Replacing this method, as the new standard for the determination of bone strength, are computational models which can interpret the entire three-dimensional arrangement of bone materials in space, and the anticipated loading patterns to predict bone strength and resistance to fracture (56).

**Peripheral Quantitative Computed Tomography**

While DXA can only directly measure BMC and derive BMD, the quality of the underlying structure of a bone is also a determining factor when quantifying bone structural integrity (57). In humans and animals, non-destructive imaging technologies such as pQCT, can differentiate between trabecular and cortical bone at a given skeletal site and provide information regarding morphological parameters with high precision (20). Recently, low-trauma fragility fractures in postmenopausal women have been associated with negative changes to trabecular and cortical microarchitecture as assessed with pQCT, but most of these associations were independent of BMD as measured with DXA (58). Although results from pQCT are powerful in their precision, and provide only a low local radiation dose, they require specialized scanners and software for the
image analyses and their measurements in humans are limited to the axial skeleton. Current commercially available pQCT scanners are able to acquire images of sufficient resolution for imaging human trabecular bone, however trabeculae of pre-clinical rodent models are substantially thinner and require higher resolutions, capable with μCT imaging, for the attainment of high quality images (59).

Micro-computed Tomography

First introduced in 1989 (60), μCT imaging is the gold standard for the direct 3-dimensional modeling and assessment of the trabecular and cortical microarchitecture in pre-clinical rodent studies (61-63). The latest μCT scanners available are able to resolve detail as low as 0.35μm (ex vivo), which provides sufficient detail for the imaging of fine trabecular structures in small rodents. Because μCT is a non-destructive method, it can be used to follow one animal longitudinally, rather than multiple animals cross-sectionally, and measure the response of bone microarchitecture to an intervention in vivo at multiple time points throughout the life course. This technology substantially reduces the number of animals required and allows for the repeated measurement of the same animal over time, which minimizes any impact of inter-animal variability that can arise with a cross-sectional study design.

Although the data obtained from repeated μCT scanning may provide powerful evidence for a change in bone health in response to an intervention within the same animal, the effect of repeated exposure to the conditions needed for proper image acquisition including exposure to radiation and to general anesthesia may be detrimental to the animal under investigation. Some studies investigating the effect of radiation exposure by repeated μCT scanning to the hind limb of rats suggest a resilience to 500
and 900 mGy radiation (64, 65), although one study of 10-month female rats found a difference between irradiated and non-irradiated controls after exposure to 400 mGy, repeated five times over 54 weeks (66). The differences observed in this study could be due to the old age of the rats, the sample size (n=4 non-irradiated controls), or because a control group rather than the contralateral limb was used for comparison (66). To our knowledge, these are the only studies of the effect of radiation exposure by µCT to the structural parameters of the tibia, all of which occur in older rats (7.5 to 10-months of age) that were no longer experiencing bone growth and modeling. Moreover, only one study assessed the effect of repeated radiation on cortical bone.

A summary of the effect of repeated radiation by µCT to bone microarchitecture of the proximal tibia in rats is included in Table 2-2. Because no study to date has followed the same rat over a 12-week study period following OVX at 12 weeks of age, this thesis investigated the effect of repeated monthly irradiation to bone structure of the proximal tibia by comparing the irradiated hind limb to the contralateral non-irradiated hind limb (Study 2). The repeated use of general anesthesia in order to minimize movement of the animal under investigation must also be considered during experimental design as this process may have negative health impacts on the animal and can become costly and time consuming for the researcher. Studies in rats have demonstrated more aversive behaviours (67), decreased cerebral blood flow (68), and learning and memory impairment in aged rats (69, 70) with repeated isoflurane administration. No studies have investigated the effect of repeated anesthesia exposure on bone health. Monitoring food intake and body weight over the duration of a longitudinal study can assess the overall health status of an animal.
Table 2-2. Summary of the effects of radiation by $\mu$CT to bone microarchitecture of the proximal tibia in rats $^1$

<table>
<thead>
<tr>
<th>Reference</th>
<th>Age (Gender), Species, Notes</th>
<th>Radiation Dose/Scan (mGy)</th>
<th>Scanner, Voxel Size</th>
<th>Scanning Frequency</th>
<th>Comparison</th>
<th>Bone Structure Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waarsing, 2006 (66)</td>
<td>10-month (F), Wistar, Sham-operated</td>
<td>400</td>
<td>SkyScan 1076, 20µm</td>
<td>Baseline, 4, 14, 34, 54 weeks</td>
<td>Non-irradiated control scanned at 54 weeks only</td>
<td>• No difference in BV/TV • SMI, Tb.Th. differ from non-irradiated limb</td>
</tr>
<tr>
<td>Brouwers, 2007 (64)</td>
<td>7.5-month (F), Wistar, retired breeder</td>
<td>993 (air)$^2$ 441 (plexiglass)</td>
<td>Scanco vivaCT 40, 15µm</td>
<td>1, 2, 3, 4, 5, 6, 7, 9 weeks</td>
<td>Contralateral limb scanned at first and final time-point</td>
<td>• No difference for any structural parameter at first or final time-point • No difference in viability of bone marrow cells</td>
</tr>
<tr>
<td>Klinck, 2008 (65)</td>
<td>8-month (F), Wistar, retired breeder, OVX and Sham-operated</td>
<td>597 (air)$^2$ 502 (plexiglass)</td>
<td>Scanco vivaCT 40, 12.5µm</td>
<td>Baseline, 2, 4, 6, 8, 12 weeks</td>
<td>Contralateral limb scanned at endpoint only</td>
<td>• No difference for any trabecular or cortical parameter in either OVX or Sham</td>
</tr>
</tbody>
</table>

$^1$ BV/TV; percent bone volume, F; female, OVX; ovariectomy, SMI; structure model index, Tb.Th.; trabecular thickness.

$^2$ Radiation dose was measured either in air or at the center of a plexiglass cylinder used to mimic the effect of soft tissue surrounding the bone in vivo on X-ray attenuation, ultimately lowering the dose of radiation experienced at the proximal tibia.
When performing μCT scanning, the preparation of the sample and the scan settings are important factors that must be considered during the experimental design as they have a profound effect on the results (Table 2-3). *In vivo* imaging of the tibia involves scanning of the whole hind limb, including fur, skin, and soft tissue surrounding the bone, which may affect the image quality and subsequent outcomes. In a study of the effect of varying thickness of water surrounding an aluminum tube used to represent bone, a greater thickness of surrounding water resulted in a reduction of beam hardening due to peripheral filtration of the X-rays away from the bone (71). But, by changing the scanning scenario from one material (aluminum) to two materials (aluminum surrounded by water), the effectiveness of the beam hardening correction software (where a single polynomial correction term is applied as in the commercial reconstruction software available from SkyScan) was reduced or eliminated. Also, different thickness of surrounding medium can change the reconstructed attenuation so that the same bone in different parts of an animal can be assigned different density values, artificially. In general, the surrounding medium complicates the task of correcting beam hardening artifact, which is necessary for the accurate quantification of bone densitometry and morphology.

One study of female Wistar rats found all trabecular morphological outcomes of the tibia, as measured by μCT at a 26μm voxel size (3-dimensional pixel), were correlated between *in vivo* and *ex vivo* scanning protocols (72), but no study has directly compared these scan protocols at a higher resolution and in an OVX rat model, where a significant loss of trabecular bone volume and structure has occurred. Study 3 of this
thesis determined how the quantification of trabecular and cortical microarchitecture of the proximal tibia differs depending on whether measured in vivo compared to ex vivo.

When performing μCT scanning, the scan settings that must be considered before image acquisition include the resolution, rotation step, exposure time, voltage and amperage, all of which can be adjusted to improve image quality and increase the signal-to-noise ratio (61). When changes to the scan settings to improve signal-to-noise ratio are made, a higher acquisition time and increased radiation dose are the tradeoffs, which must be strongly considered when performing in vivo scanning (73). In fact, doubling the resolution of a scan (halving the voxel size) causes a four-time longer scan time for which the animal must remain motionless under general anesthesia. For this thesis, μCT was used to evaluate the changes to bone outcomes in vivo and ex vivo in the rat model of postmenopausal osteoporosis, a skeletal disease characterized by compromised bone quantity and bone quality, ultimately leading to an increased risk of fragility fracture in women following the menopausal transition.
Table 2-3. Aspects to consider when designing μCT experiments

<table>
<thead>
<tr>
<th>Animal</th>
<th>Scanning Settings</th>
</tr>
</thead>
</table>
| • Exposure to radiation  
  • Exposure to general anesthesia  
  • Species  
  • Skeletal site  
  • Animal age  
  • Hormone status of the animal | • *in vivo* versus *ex vivo* sample preparation  
  • Resolution, voltage, amperage, exposure time, rotation step  
  • Image acquisition time  
  • Scan length (i.e., field of view length)  
  • Data file size |
Age-Related Bone Loss in Women

Several studies have used the 3-dimensional imaging technique, high-resolution pQCT, to quantify the age-related changes to BMD and structure in response to normal aging. In a large cross-sectional study of 373 women between the ages of 20-97 years, high-resolution pQCT of the distal radius revealed that cortical BMD remains stable until approximately mid-life, followed by a gradual decline, while declines in trabecular BMD of the lumbar spine begin in young adulthood (20-29 years) and continue throughout life (74). Longitudinal analysis of the same cohort confirmed the substantial trabecular bone loss beginning in young adulthood and continuing throughout life with acceleration during menopause (75).

Assessment of the trabecular and cortical microarchitecture provides even more information regarding the age-related changes to bone. In one cross-sectional study of 324 women (21-97 years of age), changes to trabecular bone volume (-27%), trabecular number (Tb.N.) (-13%), trabecular separation (Tb.Sp.) (+24%), cortical BMD (-22%), cortical thickness (Ct.Th.) (-52%), and endocortical area (+27%) were observed with high-resolution pQCT imaging of the wrist between the ages of 20-90 years (76). These structural changes with aging account for an increased fracture risk with age. The loss of trabeculae contributes to the biomechanical instability and an overall decrease in bone strength (77), but eventually the loss of trabecular bone stops because so few trabeculae are left to lose (78). Cortical bone loss follows trabecular loss more slowly because its surfaces are less accessible to the bone multicellular units responsible for bone remodeling (79, 80). In postmenopausal women, most cortical bone loss occurs in pores and on the endocortical surface (80). With the loss of bone (both trabecular and cortical),
the proportion of the total load carried by the cortex is increased (81). To maintain its strength and resistance to bending, the distribution of cortical bone mass is modified away from the central axis along a larger perimeter through endocortical resorption and concomitant periosteal apposition (82). In a large 7-year prospective study of over 800 women, endocortical resorption and periosteal apposition rates were measured in pre-, peri-, and postmenopausal women (83). With advancing age and menopausal status, endocortical resorption increased and periosteal apposition decreased, resulting in an overall thinning of the cortex of the distal radius and decreased resistance to bending. Fracture risk was doubled in those women with a one standard deviation lower estimate of bending strength, highlighting the clinical relevance of age-related changes to cortical bone structure (83). The loss of trabeculae, increased medullary cavity area and the changes to bone volume ultimately causes a reduction in bone mass and a compromised bone structure, predisposing a women to fragility fracture and the diagnosis of osteoporosis.

**Osteoporosis**

Osteoporosis is a disease of the skeletal system characterized by a low bone mass and compromised bone microarchitecture. Together, these outcomes result in an increased risk of fragility fracture, often resulting in increased morbidity, chronic pain and a reduced quality of life (84-88). The most common sites of osteoporotic fracture include the hip, spine, and wrist. The total annual incidence of fragility fracture in Canadian women is greater than heart attack, stroke, and breast cancer combined (1). Currently, an estimated 1.5 million Canadians aged 40 years and over have reported being diagnosed with osteoporosis (89) with at least 1 in 3 women and 1 in 5 men
suffering from an osteoporotic fracture within their lifetime (1). The diagnosis of osteoporosis is defined as a BMD, measured with DXA at the proximal femur, 2.5 or more standard deviations below the average value for premenopausal women (22). The average values, or reference ranges, used as comparative criteria for the diagnosis of osteoporosis come from the National Health and Nutrition Examination Survey (NHANES) reference database in non-Hispanic white women aged 20-29 years. Fracture risk assessment tools are also available to Canadians to estimate 10-year fracture risk, and as mentioned previously, these tools take into account other clinical risk factors.

Although osteoporosis is generally diagnosed in older individuals, it has been termed a ‘pediatric disease with geriatric consequences’ due to the importance of developing strong bones during growth and development (90). At the end of skeletal maturation when peak bone mass is reached (91) a slow and steady decline in BMD occurs. Only at the onset of menopause do women begin to lose bone mass at a faster rate than men due to the loss of endogenous estrogen production, which can lead to a diagnosis of postmenopausal osteoporosis (Figure 2-3). Peak bone mass is influenced by a variety of genetic and environmental factors including, gender, race, hormonal factors, nutrition, physical activity level, age at menarche, body weight, and other lifestyle behaviours.

Mathematical modeling using several experimental variables to predict their relative influence on the development of osteoporosis resulted in peak bone mass being the single most important factor (92). Using this model, a 10% increase in peak bone mass would delay the onset of osteoporosis by 13 years, whereas a 10% increase in the onset of menopause would delay osteoporosis by only 2 years (92). For these reasons,
lifelong habits including the intake of nutrients that support bone health is important for the early and continued bone accrual and a postponed onset of postmenopausal osteoporosis.
Figure 2-3. Age-related changes to bone mineral throughout the lifespan in females

From birth to late adolescence, the skeleton experiences rapid increases in bone mineral until the attainment of peak bone mass. At the end of skeletal maturation, a slow and steady decline in bone mass occurs until the onset of menopause in women, which coincides with a more rapid loss of bone mineral during the menopausal transition. The solid and dashed lines represent the trajectory of bone mineral throughout the lifespan when peak bone mass is attained through proper environmental factors and full genetic potential (solid line) or when inadequate environmental and genetic factors (dashed line) influence the attainment of peak bone mass. With a greater peak bone mass at young adulthood, there is more bone available to be lost before reaching a fracture threshold later in life, providing protection against fracture in late adulthood.

(Image by Longo AB, unpublished, 2016. Based on data from (93))
**Estrogen as a Mediator of Bone Turnover**

Estrogen plays an integral role in the maintenance of bone structure in adult women by maintaining a balance between bone resorption by osteoclasts and bone formation by osteoblasts (94). To preserve this balance, estrogen functions to suppress the activation frequency of bone multicellular units (95). Estrogen has direct effects on bone turnover, by inducing the differentiation of precursor cells to mature to osteoblasts (96, 97), to prevent the apoptosis of osteoblasts (98), and to induce apoptosis of osteoclasts (99, 100). To prevent bone resorption indirectly, estrogen increases OPG production (101) and reduces RANKL production (102) by the osteoblast and modulates RANK signaling in osteoclasts (103).

**Endogenous Estrogen Deficiency Leads to Bone Loss**

During the menopausal transition, endogenous estrogen levels decline substantially and this results in accelerated bone loss that has been well characterized in women (104). The greatest rate of bone loss is observed during trans-menopause (coinciding with 1 year before and 2 years after final menses) followed by a slightly decelerated rate of loss 2-5 years following final menses (104). During this period of interrupted hormone production, trabecular bone loss is 3-5 fold greater than cortical bone loss (105) due to an increased activation frequency of bone multicellular units on the trabecular surfaces with an imbalance that favours bone resorption (106). This imbalance occurs mainly because of an increase in osteoclast formation, activation, and lifespan with the loss of endogenous estrogen production (107).
OVX Rat Model of Postmenopausal Osteoporosis

Studies of bone health in humans rely mainly on self-reported fracture occurrence and surrogate markers such as BMD by DXA and serum biochemical markers because of the obvious limitations with obtaining bone biopsies from human subjects. The use of animal models however allows for the timely and direct measure of changes to bone quantity and bone quality in response to specific interventions. The OVX rat is a well-established animal model, which results in the rapid loss of bone mass and bone strength followed by a slower more gradual rate of bone loss following the surgical removal of the ovaries, similar to the changes observed in human bone during and following the menopausal transition (108-114). Similar to the response in humans, bone multicellular unit-based remodeling causes bone loss on the endocortical surfaces following OVX in rats (115, 116), making this an appropriate model for the study of the pathogenesis and progression of osteoporosis.

The development of in vivo imaging techniques have allowed for the longitudinal investigation of the impact of OVX on the microarchitecture of skeletal-specific sites in the rat model of osteoporosis.

Site-Specific Effect of OVX

OVX causes a highly reproducible skeletal response involving site-specific bone loss and the subsequent development of osteoporosis (108, 110, 111, 113, 117). Following OVX, the proximal tibia is the first skeletal site to experience changes to trabecular bone volume after only 8-14 days following surgery (113, 117, 118). This site also experiences the earliest reduction in BMD and deterioration in trabecular microarchitecture compared to other skeletal sites (119). The trabecular bone of the
femur and lumbar vertebrae (LV) experience changes similar to the proximal tibia, but at slower rates (109, 119, 120). In one of the most comprehensive studies of the site-specific effect of OVX in 6-month SD rats, the long bones (femur and tibia) showed the largest changes from baseline in most trabecular microarchitecture outcomes (121). The LV showed similar patterns of bone loss 36-weeks post-OVX (121). While these bones experience similar overall patterns of loss following OVX at 6-months of age, the rate of these changes is different. In general, OVX caused significant and sustained reductions to trabecular BMD, percent bone volume (BV/TV), and Tb.N. after only 4 weeks and 12 weeks (24 weeks for Tb.N. reductions) in the long bones and LV, respectively. Tb.Sp. of the long bones was increased in only 12-weeks post-OVX and 24-weeks in the LV.

Varying rates of bone loss between the proximal tibia, proximal and distal femur, and LV has been supported by other comprehensive, cross-sectional studies of the rat skeleton in response to OVX (119). Together, these findings confirm a systemic effect of OVX, but that the resulting outcome of this effect is not equal across all skeletal sites. Therefore, I determined the changes to bone microarchitecture at multiple skeletal sites to fully establish the skeletal response to an intervention (Study 4).

Similar to the changes to cortical bone experienced in humans following menopause, OVX causes changes to Ct.Th. of the long bones at a slower rate than changes observed in trabecular bone (110). This is because as bone is being resorbed from the endocortical surfaces (interior), it is being formed on the periosteal surface (outer) (110, 122-125). For this reason, an increase in the medullary area (Ma.Ar.) should be considered the most sensitive measure of cortical bone loss in the OVX rat
(110), rather than a change in Ct.Th. The earliest changes to Ma.Ar. following OVX occur in the mid-shaft of the tibia (126).

**Longitudinal Changes to Bone Following OVX**

To characterize the changes to bone quantity and bone quality, several cross-sectional and longitudinal studies have been conducted in the preclinical model of postmenopausal osteoporosis and have been summarized in Table 2-4 and the findings are discussed below.
Table 2-4. Longitudinal changes to bone following OVX

<table>
<thead>
<tr>
<th>Reference</th>
<th>Age at OVX (Strain)</th>
<th>Experimental Timeline</th>
<th>Skeletal Site</th>
<th>Bone-Related Outcome of OVX rats (Compared to)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DXA, Strength Testing, Histology, Biomarkers</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Bagi, 1997 (127)          | 4-month (SD)        | 0, 4, 8, 12 weeks    | Femur Neck    | • Decrease BMD (DXA) by 4 weeks (Sham)  
• Decrease ultimate strength and stiffness by 4 weeks (Sham)  
• Increase trabecular mineralized surface (%), bone formation rate, Tb.Sp. by 4 weeks (Sham)  
• Decrease Tb.Th., Tb.N. by 4 weeks (Sham)  
• Increase periosteal and endocortical mineralized surface (%) and bone formation rate by 4 weeks (Sham) |
| Zhang, 2007 (128)         | 13-week (Wistar)    | 0, 4, 18 weeks       | Femur Diaphysis| • No change in any bone mechanical properties (Sham)                                                                                                                                                                                                                  |
| Campbell, 2008 (118)      | 6 to 8-month (Wistar) | 0, 2, 4, 6, 8, 12 weeks | Femur Midpoint, LV4 | • Decrease ultimate stress (femur) (Sham)  
• Decrease ultimate load (LV4) (Sham)                                                                                                                                                                                                                             |
| Abuohashish, 2015 (129)   | 3-month (Wistar)    | 7 weeks              | Distal Femur  | • Decrease calcium and phosphorous content (Sham)  
• Increase all formation and resorption markers (Sham)                                                                                                                                                                                                               |
| **Microarchitecture**     |                     |                       |               |                                                                                                                                                                                                                                                                     |
| Lane, 1998 (117)          | 6-month (SD)        | 0, 5, 8, 13, 16, 29, 33, 42, 50 days | Proximal Tibia | • Decrease connectivity by 5 days (Baseline)  
• Decrease BV/TV by 8 days (Baseline)  
• Increase Tb.Sp. (Sham)  
• Decrease Tb.N. (Sham)  
• Increase bone formation and resorption markers (Sham)                                                                                                                                                                                                       |
| Laib, 2001 (113)          | 6-month (SD)        | 0, 12, 35, 60, 110 days | Proximal Tibia | • Decrease in BV/TV, connectivity, Tb.Th., and Tb.N. by 12 days (Baseline)  
• Increase Tb.Sp. at 110 days (Sham)                                                                                                                                                                                                                           |
<table>
<thead>
<tr>
<th>Study</th>
<th>Age/Duration</th>
<th>Timepoints</th>
<th>Region</th>
<th>Observations</th>
</tr>
</thead>
</table>
| Waarsing, 2004 (130)          | 10-month (Wistar) | 0, 4, 14 weeks | Proximal Tibia | • Decrease BV/TV, Tb.Th. by 4 weeks (Baseline)  
• Increase periosteal resorption, endocortical apposition, and Ct.Th. (Sham) |
| Waarsing, 2006 (66)           | 10-month (Wistar) | 0, 4, 14, 34, 54 weeks | Proximal Tibia | • Decrease BV/TV and connectivity by 4 weeks (Baseline)  
• Increase Tb.Th. by 14 weeks (Baseline)  
• Increase periosteal resorption, endocortical apposition, and Ct.Th. (Sham) |
| Boyd, 2006 (131)              | 8-month (Wistar) | 0, 1, 2, 3, 4, 5, 6 months | Proximal Tibia | • Decrease BV/TV, Tb.N., Tb.Th. by 4 weeks (Baseline)  
• Increase Tb.Sp. by 4 weeks (Baseline) |
| Zhang, 2007 (128)             | 13-week (Wistar) | 0, 4, 18 weeks | Proximal Tibia | • Decrease trabecular BMD by 4 weeks (Sham)  
• No change in cortical outcomes (Sham) |
| Campbell, 2008 (118)          | 6 to 8-month (Wistar) | 0, 2, 4, 6, 8, 12 weeks | Proximal Tibia | • Decrease BV/TV, Tb.N., Tb.Th., and connectivity by 2 weeks (Baseline)  
• Decrease Tb.Sp. by 4 weeks (Baseline)  
• Increase Ct.Th. by 6 weeks (Baseline) |
| Klinck, 2008 (65)             | 8-month (Wistar) | 0, 2, 4, 6, 8, 12 weeks | Proximal Tibia | • Decrease BV/TV, Tb.N., Ma.Ar. over 12 weeks (Baseline)  
• Increase Tb.Sp., Ct.Th., Ct.Ar. over 12 weeks (Baseline)  
• No change in Tb. Th. over 12 weeks (Baseline) |
| Francisco, 2011 (119)         | 3-month (Wistar) | 2, 5, 10, 15, 20, 25, 30 weeks | Proximal Tibia | • Decrease BMD, Tb.N. by 5 weeks (Sham)  
• Increase Tb.Sp. by 5 weeks (Sham)  
Distal Femur | • Decrease BMD by 15 weeks, decrease Tb.N. by 10 weeks (Sham)  
LV | • Decrease BMD by 20 weeks (Sham)  
• Decrease Tb.N. by 10 weeks (Sham) |
|                              | 5-month (Wistar) | 2, 5, 10, 15, 20, 25, 30 weeks | Proximal Tibia | • Decrease BMD, Tb.N. by 2 weeks (Sham)  
• Increase Tb.Sp. by 10 weeks (Sham)  
Distal Femur | • Decrease BMD by 15 weeks, decrease Tb.N. by 20 weeks (Sham)  
LV | • Decrease BMD by 10 weeks, decrease Tb.N. by 15 weeks (Sham) |
<table>
<thead>
<tr>
<th>Study</th>
<th>Bone Model</th>
<th>Duration</th>
<th>Time Points</th>
<th>Tibia</th>
<th>Changes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shin, 2012 (132)</td>
<td>Proximal Tibia</td>
<td>11-month (Wistar)</td>
<td>2, 5, 10, 15, 20, 25, 30 weeks</td>
<td>Decrease BMD by 2 weeks, decrease Tb.N. by 5 weeks (Sham)</td>
<td>Increase Tb.Sp. by 15 weeks (Sham)</td>
</tr>
<tr>
<td>Shin, 2012 (132)</td>
<td>Distal Femur</td>
<td>3-month (SD)</td>
<td>4, 8 weeks</td>
<td>Decrease BMD by 10 weeks, decrease Tb.N. by 15 weeks (Sham)</td>
<td></td>
</tr>
<tr>
<td>Shin, 2012 (132)</td>
<td>LV</td>
<td>3-month (SD)</td>
<td>4, 8 weeks</td>
<td>Decrease BMD by 25 weeks, decrease Tb.N. by 20 weeks (Sham)</td>
<td>Increase Tb.Sp. by 20 weeks (Sham)</td>
</tr>
<tr>
<td>Liu, 2015 (121)</td>
<td>Femur</td>
<td>6-month (SD)</td>
<td>0, 2, 4, 12, 24, 36 weeks</td>
<td>Decrease BMD, BV/TV, Tb.N. (Sham)</td>
<td>Increase Tb.Th., Tb.Sp. by 8 weeks (Sham)</td>
</tr>
<tr>
<td>Liu, 2015 (121)</td>
<td>LV</td>
<td>6-month (SD)</td>
<td>0, 2, 4, 12, 24, 36 weeks</td>
<td>Decrease BMD, BV/TV, Tb.N., Tb.Th. (Sham)</td>
<td>Increase Tb.Sp. by 8 weeks (Sham)</td>
</tr>
<tr>
<td>Hatori, 2015 (133)</td>
<td>Distal Femur, Proximal Tibia</td>
<td>3-month (SD)</td>
<td>4, 14 days</td>
<td>Decrease BMD, BV/TV, Tb.N., Tb.Th., by 4 weeks (Sham)</td>
<td>Increase Tb.Sp. by 12 weeks (Sham)</td>
</tr>
<tr>
<td>Hatori, 2015 (133)</td>
<td>LV</td>
<td>3-month (SD)</td>
<td>4, 14 days</td>
<td>Decrease Tb.Th. at 4 weeks, only (Sham)</td>
<td></td>
</tr>
<tr>
<td>Abuohashish, 2015 (129)</td>
<td>Proximal Tibia</td>
<td>3-month (Wistar)</td>
<td>7 weeks</td>
<td>Decrease BMD, BV/TV, Tb.N., Ct.Th. (Sham)</td>
<td>Increase Tb.Sp., Ec.Pm., Ma.Ar. (Sham)</td>
</tr>
<tr>
<td>Abuohashish, 2015 (129)</td>
<td>Distal Femur</td>
<td>3-month (Wistar)</td>
<td>7 weeks</td>
<td>Decrease BMD, BV/TV, Tb.Th., Tb.N., Ct.Th., Ct.Ar. (Sham)</td>
<td>Increase Tb.Sp., Ec.Pm., Ps.Pm. (Sham)</td>
</tr>
</tbody>
</table>

1 BMD; bone mineral density, BV/TV; percent bone volume, Ct.Ar.; cortical area, Ct.Th.; cortical thickness, DXA; dual energy X-ray absorptiometry, Ec.Pm., endocortical perimeter, LV; lumbar vertebrae, Ma.Ar.; medullary area, OVX; ovariectomy, Ps.Pm.; periosteal perimeter, SD; Sprague-Dawley, Tb.N.; trabecular number, Tb.Sp.; trabecular separation, Tb.Th.; trabecular thickness.
Changes to Bone Mass, Structure, and Strength Following OVX

The first studies to investigate the longitudinal change in BMD, derived structural indices, and strength following OVX used DXA, histomorphometry, and biomechanical strength testing, respectively and have provided contradicting results (Table 2-4). Significant reductions in BMD, ultimate strength, and trabecular thickness (Tb.Th.) and Tb.N. in the femur neck occurred following only 4-weeks post-OVX (127). This study also observed increases in periosteal, endocortical and trabecular mineralized surface with concomitant increases in bone formation rates suggesting an increased rate of bone turnover following OVX at the femur neck (127). The mechanical properties of ultimate stress and ultimate load have been shown to decrease in response to OVX in the femur midpoint and LV, respectively (118). In contrast, bone mechanical properties of the femur midpoint were not affected 4- or 18-weeks post-OVX compared to baseline values (128). To resolve these conflicting results, higher-resolution imaging using μCT has been used to more fully characterize the longitudinal changes to bone following OVX in the rat model of postmenopausal osteoporosis.

Changes to Bone Microarchitecture Following OVX

The earliest deterioration to trabecular bone connectivity and bone volume following OVX was observed after only 5 and 8 days, respectively, in the right tibia of the 6-month old SD rat (117). In this model, OVX caused a rapid phase of bone loss in the first 5-8 days followed by a slower, steadier decline up to 50 days post-OVX. The rapid deterioration of trabecular bone tissue followed by a slow decline has been observed in other, similar longitudinal studies (66, 113, 118, 128, 130-132). Generally, bone volume and Tb.N. is decreased (65, 113, 117-119, 121, 129, 131-133) and Tb.Sp. is
increased (65, 113, 117, 118, 121, 129, 131-133) as early as 2- and 4-weeks following OVX, respectively. These changes have been suggested to be due to an imbalance in bone turnover as both bone formation and resorption markers are elevated in OVX rats compared to Sham-control (117, 118, 128, 129, 132).

Of particular interest is the rate and direction of change of Tb.Th. following OVX surgery. Some studies report increases (66, 133), no change (65, 117), or sustained declines (129), but most have reported an initial decrease in Tb.Th. that eventually plateaus (113, 118, 121, 131). The time at which this plateau is reached varies from 2 weeks (118) to 6 months (131). Changes to Tb.Th. also seem to be site-specific, as the LV experiences declines earlier than the tibia and femur (121). At the cortex of the proximal tibia, resorption at the periosteal surfaces and apposition at the endocortical surfaces result in net increases in Ct.Th. (118, 130) and these changes can occur for up to 34-weeks post-OVX (66). OVX causes significant declines in Ct.Th., cortical area (Ct.Ar.), and increases in Ma.Ar., endocortical perimeter (Ec.Pm.) and periosteal perimeter (Ps.Pm.) of the tibia compared to baseline and Sham values (65, 129, 133). In the femur and LV, decreases in cortical BMD compared to Sham have also been observed (132). However, analysis of the tibia diaphysis by ex vivo µCT 4- or 18-weeks following OVX did not reveal changes compared to Sham (128). Changes to the cortical bone proceed slower than on the trabecular surfaces, which is a possible reason for these discrepancies. Very few studies have investigated the changes to cortical microarchitecture including the indices, Ma.Ar., Ps.Pm., Ec.Pm., Ct.Th., and cortical area fraction (Ct.Ar./Tt.Ar), which together provide a more comprehensive understanding of any hormone-related changes to structure. This thesis more thoroughly
investigated the changes to cortical bone following OVX at 3 months (Study 1 and 4) by quantifying a wide-range of cortical and trabecular parameters.

Most studies assessing the longitudinal effect of OVX on bone microarchitecture have used an aged model (≥6 months at time of OVX), but the long-term impact of undergoing OVX during periods of modeling and growth (3 months of age), as was done in this thesis, have not been as extensively studied in the rat. Three months of age is a common time point used in studies of nutritional interventions in the OVX rat model (134-141), and so the longitudinal impact of OVX on bone microarchitecture at this stage of bone modeling was characterized. Studies of older rats are less common in the literature because of the higher cost and longer study periods associated with aged rats. Rats over 3-months of age are substantially more expensive when ordering from a large supplier, such as Charles River Canada, than younger (<3 months of age) rats. Receiving young rats from a supplier and aging them on-site is also costly and an inefficient use of time and resources. Many of the studies of older rats in the literature (>8 months of age) involve retired breeders. Often poor records, involving the exact age and the number of pregnancies (and number of pups in a litter), are kept by the supplier, which provide many uncertainties and limitations in bone characterization studies. Pregnancy and lactation cause significant changes to the skeleton in the rat, but the bone formation that occurs during the period after pregnancy and lactation does not restore the bone mass to that of age-matched nulliparous control rats (142). For all of these reasons, studying rats having undergone OVX at 3-months of age is a practical and cost-effective solution for researchers. Therefore, further studies for the characterization of the changes to bone microarchitecture were necessary (Study 1).
Effect of OVX on Food Intake, Body Weight, and Body Composition

Following OVX, rats experience a transient hyperphagia for roughly 6 weeks following surgery (143). This transient increase in food intake compared to Sham-operated rats causes the characteristic increase in body weight seen following OVX. The higher body weight is usually maintained past study endpoints (143). Along with this increase in body weight, body composition is altered with OVX and rats experience significant increases in fat mass compared to their Sham-counterparts (143). While food intake, body weight, and body composition are altered with OVX-surgery, these factors do not modulate bone mass, BMD, or biomechanical strength of the femur or LV 14-weeks post-OVX (143). Changes in trabecular bone mineral and microarchitecture of the proximal tibia and distal femur are also maintained despite weight differences in Sham compared to OVX rats (119). Therefore, increased weight gain and fat mass as a result of transient hyperphagia induced by OVX do not modulate the estrogen-deficiency induced bone loss in OVX rats.

Strengths and Limitations of the OVX Rat Model of Postmenopausal Osteoporosis

The strengths and limitations of the OVX rat model of postmenopausal osteoporosis are outlined in Table 2-5 and are discussed below.

Strengths

There are several advantages to utilizing the OVX rat as a model for postmenopausal osteoporosis. For example, the response at the proximal tibia, distal femur, and LV mimics the loss that occurs in postmenopausal women making it the appropriate model for investigating the pathogenesis of osteoporosis (144). In fact, the OVX rat is the Food and Drug Administration-approved model for the evaluation of
agents used in the treatment or prevention of postmenopausal osteoporosis (144). There is also literature regarding the characterization of the model and the longitudinal effect of OVX on bone quantity and bone quality, but as discussed previously, most work has been done when OVX occurs at 6-months of age or greater. The OVX rat model provides researchers with a time and cost-effective solution to investigating the effects of various interventions on bone health. In the rat and the human, the average remodeling cycle is roughly 40 and 200 days, respectively (144, 145). Therefore, a 12-week trial in the OVX rat (a typical study duration) results in approximately two complete remodeling periods, which is roughly equal to a 1.5 year clinical trial in humans. The rat model of osteoporosis also provides the advantage of being able to undergo repeated in vivo high resolution imaging at any skeletal site (central or axial), although the effect of repeated radiation exposure to the quality of the bone is not well understood.

Limitations

The OVX rat model does have some limitations to consider, the first of which is the lack of Haversian systems in cortical bone (146). Haversian systems are concentric rings surrounding blood vessels that increase the porosity of cortical bone and function as areas of bone remodeling (147). Without Haversian systems in the rat model, effects of various interventions on cortical remodeling and cortical porosity cannot be fully extrapolated to the human skeleton. Also, linear growth of the long bones of the rat continues longer than their human equivalent. In female SD rats, the growth of the tibia is rapid until 6 months, declines up to 12 months, and growth ends after 18 months (115). The epiphyseal growth plate does not fuse until after 6.5-months of age (148). Another difference between the OVX rat model and humans involves the weight-distribution on
skeletal sites. Humans are bipeds whereas rats are primarily quadrupeds, altering in which direction and the relative load applied to common sites of fracture including the wrist, LV, femur neck, and tibia.

Although there is a multitude of literature available on the OVX rat model, there is no consensus surrounding the ideal age of the rat at OVX. Several authors have suggested performing OVX at 9-months of age or later, coinciding with the attainment of peak bone mass (110, 124, 149), although some authors have suggested OVX as early as 6 weeks of age is sufficient (150). One study investigating the relationship between age and time post-OVX on bone mineral and trabecular microarchitecture performed either Sham-control or OVX surgery on Wistar rats at 12, 24, and 44 weeks of age and subsequently sacrificed these rats at intervals over 30 weeks post-surgery (119). Although OVX caused significant reductions in BMD compared to Sham, authors of this study found increases in bone mineral up to 10-weeks post-surgery in the rats having undergone OVX at 12 weeks of age, suggesting they were still experiencing growth and that OVX inhibited this growth rather than inducing bone loss at the proximal tibia (119). Therefore, older Wistar rats (6 through 9 months of age) are ideal when studying the skeletal response to OVX, but as previously discussed, the high cost associated with ordering and maintaining rats older than 3-months of age is a major limitation for many researchers. To avoid the issue of continued growth in the 3-month OVX rat model, measurements of the trabecular bone should be taken at least 1mm distal to the growth plate (124).
Table 2-5. Strengths and limitations of the OVX rat model

| Strengths | • Similar skeletal response to loss of estrogen production as occurs in postmenopausal osteoporosis  
|          | • FDA-approved model of postmenopausal osteoporosis  
|          | • Characterization of the model (when OVX is performed at 6 months)  
|          | • Time and cost effective alternative to human trials  
|          | • Ability to quantify outcomes not available in human trials  
| Limitations | • Lack of Haversian canals may limit applicability of findings in cortical bone  
|           | • Extended periods of linear growth and epiphyseal growth plate fusion of the long bones  
|           | • Altered load distribution on skeletal sites (quadruped versus biped)  
|           | • Lack of consensus on the ideal age to perform OVX  
|           | • Immediate loss of endogenous estrogen production compared to slow transition in the human  

1 FDA; Food and Drug Administration, OVX; ovariectomy
Dietary interventions have the potential to modulate bone health in the OVX rat. Most studies of nutritional interventions however, involve introduction to the nutrient following OVX as a treatment strategy. But, very few studies have investigated a dietary intervention as a preventative strategy by introducing the dietary intervention prior to OVX. *This thesis investigated the potential protective effect of PUFA on bone health by altering the PUFA content of the diet of 1-month SD rats for 2 months prior to OVX at 3-months of age, and following these rats through to 6-months of age (Study 4).*

**Polyunsaturated Fatty Acids**

The fatty acids that are found in lipid molecules are the major components of dietary fats. Once in the body, fatty acids are oxidized for fuel or incorporated in blood lipids, fat deposits, and as the structural unit of cell membranes. The resultant health effects of fat and fatty acids depend largely on the saturation properties of the fatty acids. Fatty acids exist in varying chain lengths, ranging from 4 to 30 carbon atoms and can be saturated (contain no double bonds), monounsaturated (contain 1 double bond), or polyunsaturated (contain more than 1 double bond). In almost all naturally occurring PUFA, the double bond is found on either the 3rd, 6th, or 9th carbon atom from the terminal methyl group, deeming them n-3, n-6, or n-9 series PUFA.

**Food Sources and Recommended Intakes of PUFA**

The n-6 and n-3 PUFA series are of major importance because of their known impact on health. Linoleic acid (LA, 18:2n-6), and alpha-linolenic acid (ALA, 18:3n-3) cannot be synthesized in the body and thus are called the essential fatty acids. Through a process of desaturation and elongation, LA and ALA can be converted to the longer chain PUFA, arachidonic acid (AA, 20:4n-6) or eicosapentaenoic acid (EPA, 20:5n-3) and
docosahexaenoic acid (DHA, 22:6n-3), respectively. LA is found in very high proportions in many vegetable oils, including sunflower, safflower, soybean and corn oils and is the predominant PUFA consumed in Western diets (151). ALA contributes approximately 10% of the total PUFA intake in Western diets and is found in high concentrations in flaxseed, perilla oil, walnuts, and many n-3 fortified food products (Table 2-6).

ALA can be converted by desaturase and elongase enzymes to longer chain n-3 PUFA, but this conversion is relatively inefficient, with only 21% and 9% of ALA converted to EPA and DHA, respectively (152). While increasing ALA intake has been shown to increase EPA concentrations, only by simultaneously reducing LA intake can DHA status be increased (153). Therefore, increasing ALA intakes may not be an effective strategy for increasing concentrations of the longer chain n-3 PUFA in the body. This also indicates that the health benefits that have been associated with higher intakes of ALA are not likely due to the downstream activity of their long chain derivatives, EPA or DHA. Fatty fish such as salmon, herring, mackerel, sardines, and smelt are good sources of EPA and DHA and incorporating these foods in the diet is the best way to increase EPA and DHA in the body.

Currently, Health Canada recommends an adequate intake for LA and ALA for women and men (154) (Table 2-6). Based on levels in a 100 g serving (half a cup), safflower and sunflower oil provide approximately 74 g and 65 g of LA, respectively. Vegetable oil and soybean oil provide 57 g and 50 g of LA in a 100g serving. A 100 g serving of flaxseed oil will provide approximately 53 g of ALA, while canola oil and chia seeds provide 9 g and 17 g of ALA, respectively. Therefore, roughly one 15 g serving of
safflower oil and only 500 mg of flaxseed oil provides the recommended daily adequate intake for LA and ALA, respectively, for women over 50 years of age. Although no recommendations exist for AA or the long chain n-3 PUFA, EPA and DHA, it is suggested that up to 10% of the acceptable macronutrient distribution range for n-3 PUFA can be consumed as EPA and/or DHA. Based on an 85 g serving, salmon, herring, and mackerel provide roughly 1.8, 1.7, and 1.6 g EPA+DHA, respectively. Shellfish and canned tuna provide roughly 0.2 g EPA+DHA in an 85 g serving. Therefore, 10% of the acceptable macronutrient distribution range for n-3 PUFA can be consumed as 1 to 2 servings of fish per week, depending on the type of fish consumed.

Because LA and ALA compete for the same pool of elongase and desaturase enzymes for conversion to their longer chain PUFA and their subsequent metabolites, the ratio of n-6 to n-3 intake is of particular importance. The excess of LA in the average Western diet results in ratios as high as 15 through 17:1 in the American population (155), although ratios from 7 through 8:1 are consumed by Canadians (89). The discrepancy in LA to ALA intakes in North American populations is most likely due to the primary oil used in each country. Soybean oil (high in LA) is the predominant oil used in USA while canola oil, which provides less LA, is the predominant oil used in Canada (89). Excess n-6 PUFA intake can lead to a state of chronic inflammation in the body through production and downstream signaling of PUFA-derived molecules.
Table 2-6. Adequate intakes and common sources of LA (n-6), ALA (n-3), and EPA and DHA (n-3) recommended by Health Canada

<table>
<thead>
<tr>
<th></th>
<th>Females</th>
<th>Males</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9-13y</td>
<td>14-18y</td>
</tr>
<tr>
<td><strong>LA (n-6)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-10% of energy</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td><strong>Sources</strong></td>
<td>• Safflower oil, sunflower oil, vegetable oil, soybean oil, corn oil, margarine, salad dressings</td>
<td></td>
</tr>
<tr>
<td><strong>ALA (n-3)</strong></td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td>0.6-1.2% of energy</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sources</strong></td>
<td>• Canola oil, flaxseed oil, perilla oil, walnuts, chia seeds</td>
<td></td>
</tr>
<tr>
<td><strong>EPA/DHA (n-3)</strong></td>
<td>0.1</td>
<td>0.11</td>
</tr>
<tr>
<td>Up to 10% AMDR of n-3 PUFA can be consumed as EPA and/or DHA</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sources</strong></td>
<td>• Fatty fish such as salmon, herring, mackerel, sardines, shellfish, and fish oils</td>
<td></td>
</tr>
</tbody>
</table>

1 ALA; alpha-linolenic acid, AMDR; acceptable macronutrient distribution range, DHA; docosahexaenoic acid, EPA; eicosapentaenoic acid, LA; linoleic acid; y; year
Mechanisms of Action of PUFA on Bone Health

Although there is no single mechanism that explains the actions of n-6 and n-3 PUFA, there are several potential physiological processes that affect bone health either directly or indirectly. Studies of calcium balance have reported an increase calcium absorption in the small intestine (156-159) and a decrease in calcium excretion (160, 161) with n-3 PUFA supplementation in animal models. These studies support the direct effect of dietary PUFA on bone metabolism by increasing the availability of calcium in the body.

Dietary PUFA directly affect mesenchymal stem cell differentiation, which have the genetic potential to differentiate into adipocytes or osteoblasts, through activation of the peroxisome-proliferator activator receptor (PPAR) family of transcription factors (162). The n-6 PUFA, AA, is a potent stimulator of adipogenesis, whereas, EPA and DHA have benefits to osteoblastogenesis (162). Therefore, n-3 PUFA directly influence bone metabolism through the differentiation and increased expression of the osteoblast.

The literature is replete with evidence supporting the incorporation of dietary PUFA into the cell membranes as phospholipids in various tissue types. Bone fatty acid composition has been extensively studied after dietary supplementation in animal models and the composition of the diet is generally well reflected in the bone fatty acid composition (139, 141, 163-170). PUFA incorporated into the membrane as phospholipids can be cleaved, releasing the longer chain PUFA – primarily AA, EPA and DHA – substrates of the enzymes cyclooxygenase (COX) (constitutive COX-1 and inducible COX-2), and lipoxygenase (LOX) for the production of small signaling molecules called eicosanoids (Figure 2-4). AA and EPA are the key regulators of
inflammation in the body. The eicosanoids derived from AA are generally considered to be pro-inflammatory in nature, whereas EPA-derived eicosanoids are less inflammatory or even anti-inflammatory (171). However, the overall physiological outcome of these regulators depends upon the nature and timing of the eicosanoid production, the concentration of the eicosanoids, and the sensitivity of the target cells to the eicosanoid present (172).

PUFA of the n-6 and n-3 series are in direct competition for these key conversion enzymes, but COX-1 exhibits the greatest specificity for AA, and so the 2-series prostaglandin (PGE$_2$) is preferentially produced (173). PGE$_2$ has biphasic properties and can act as a modulator of bone formation at low concentrations (174) and bone resorption at high concentrations in human cell culture (175). Prostaglandins and their downstream metabolites can influence the expression and activity of growth factors (i.e., TGF-$\beta$, IGF-1), cytokines (i.e., IL-1, IL-6, IL-10, TNF-$\alpha$), and transcription factors (i.e., NF$\kappa$B, PPAR), having further indirect effects on bone metabolism (176). The long chain n-3 PUFA, EPA and DHA, are substrates for the production of 3-series of prostaglandins, thromboxanes, and resolvins, all generally considered to have anti-inflammatory properties.

Although it has been suggested that AA, EPA, and DHA elicit their inflammatory effects in the body through downstream signaling of their respective eicosanoids, the physiological effect of LA and ALA are likely independent of these signaling molecules since their conversion rate to longer chain PUFA is low.
Figure 2-4. Metabolism of the essential fatty acids: LA and ALA

N-6 (yellow) and n-3 (blue) PUFA compete for the same pool of elongase, desaturase, COX, and LOX enzymes. AA, EPA, and DHA are substrates for the production of eicosanoids generally considered to be pro-inflammatory (red) and anti-inflammatory (green) in nature, respectively.

(Image by Longo AB, adapted from (3))
PUFA and Bone Cells

The impact of PUFA and their derivatives on the osteoblast and osteoclast have been extensively studied. PUFA metabolism and their impact in vitro have been summarized in Figure 2-5 and are discussed below.

Effects of PUFA on the Osteoblast

Several studies have investigated the effects of PUFA on the proliferation, differentiation, and rate of activity of cells of the osteoblast lineage. In 2003, Watkins et al. reported the expression of the earliest and most specific marker of osteoblastogenesis (177), Runx2 was stimulated by AA, LA, and EPA in mouse calvarial osteoblasts, and that the rate of expression was time- and dose-dependent (178). While both n-3 and n-6 PUFAs were able to stimulate Runx2 expression, EPA-treatment resulted in a greater bone alkaline phosphatase activity, a marker of bone formation, compared to treatment with the n-6 PUFA, AA (178). Similar studies have demonstrated an increased expression of certain pro-inflammatory cytokines in a time- and dose-dependent manner in response to AA-treatment (179, 180) but that this effect can be inhibited by treatment with EPA (180). This could be due to the opposing inflammatory and anti-inflammatory effects of the prostaglandin derivatives of AA and EPA, respectively (171). Both AA and DHA have been found to cause a dose-dependent inhibition of the proliferation of osteoblast cells in vitro (181, 182), but this inhibition is thought to be through different mechanisms, as incubation with AA caused an increase in PPAR-γ expression that was not found with DHA incubation (183).

Exposure of the murine osteoblast cell line to AA and DHA at varying concentrations and varying lengths of time in vitro, resulted in a dose- and time-
dependent response of bone alkaline phosphatase activity (184). With only acute exposure (48 hours), increasing concentrations of DHA caused a decrease in bone alkaline phosphatase activity, whereas with chronic exposure (14 days) increasing concentrations of AA caused a decrease in activity (184). The current dietary patterns of Western countries suggest a chronic excess of n-6 PUFA intake with n-6 to n-3 PUFA ratios from 15 through 17:1 (155). As a consequence, the resulting chronic exposure of n-6 PUFA in the body may cause detrimental effects to osteoblast cells and to osteoblast differentiation and maturation. Together, these results highlight the complexity of timing, dose, and PUFA source on the function of the osteoblast.

Effects of PUFA on the Osteoclast

Osteoclastogenesis is primarily controlled by the RANKL/RANK/OPG triad (185). In mouse calvarial osteoblasts, treatment with the highest concentration of AA or DHA (20 µg/ml) caused a decrease in OPG secretion in the surrounding media (186). AA treatment also caused increased secretion of the soluble RANKL, although statistical testing could not be performed because the control cells produced RANKL concentrations lower than the detectable limit of the assay. AA ultimately lowered the ratio of OPG to soluble RANKL, but whether this change was sufficient to increase osteoclastogenesis and ultimately lead to enhanced bone resorption was not investigated (186). As a supportive follow-up to this study, treatment of mouse osteoblast-like cells with the n-6 PUFAs, γ-linoleic acid and AA, caused an increased percentage of RANKL-expressing cells compared to cells treated with the long chain n-3 PUFAs, EPA and DHA (187). AA decreased OPG secretion by 20%, but γ-linoleic acid, EPA, and DHA did not have an effect (187). These studies support the role of n-6 PUFA in stimulating
osteoclastogenesis through the RANKL/RANK/OPG biochemical marker triad. In primary murine bone marrow cell culture, EPA, DHA, or their combination inhibited tartrate resistant acid phosphatase staining, a marker of osteoclast maturation, compared to LA, AA, or their combination (188), further supporting the role of n-6 PUFA as a mediator of osteoclastogenesis and osteoclast activity.

The study of cell culture models allows a researcher to ask and to answer specific questions without the influence of other systems. While this technique allows for the investigation into the mechanisms responsible, studies of humans and animal models provide insight into how diet can affect changes at the organ and whole-body level, ultimately affecting overall health.
Figure 2-5. Concentration-dependent effects of LA, AA, EPA, and DHA on bone health outcomes in cell culture and animal models

A dose-dependent effect of treatment with AA, and DHA has been observed with serum or urinary markers of bone formation and resorption and on bone cell proliferation and activity. AA concentrations can be increased with increasing dietary ratios of n-6 to n-3 PUFA. Higher concentrations of AA and DHA have been linked to increased markers of bone resorption and lower concentrations of AA has been linked to markers of bone formation. Large inverted triangles are used to represent dose-dependent effects of AA and DHA. Serum or urinary markers outlined in green and red are generally considered to be either bone formation or bone resorption markers, respectively. (Image by Longo AB, unpublished, 2016)

BALP; bone alkaline phosphatase, OC; osteoclast, OPG; osteoprotegerin, RANKL; receptor activated of nuclear factor kappa beta ligand, TRAP; tartrate resistant acid phosphatase
PUFA and Human Studies

Multiple large observational studies and randomized controlled trials (RCT) have investigated the associations between dietary intake patterns and bone-related health outcomes. Specifically, individual fatty acid intake, ratio of fatty acid intake, or fish consumption has been quantified and compared to either the risk of fragility fracture, BMD, biochemical markers, or a combination of these outcomes to identify associations in women. A summary of the findings from these studies is outlined in Table 2-7 and is discussed below.
Table 2-7. Summary of findings from observational studies concerning PUFA intake and bone health

<table>
<thead>
<tr>
<th>Reference</th>
<th>Population Characteristics</th>
<th>PUFA Intake</th>
<th>Bone-Related Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cross-Sectional Studies</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weiss, 2005 (189)</td>
<td>564 PM women, Caucasian American, Rancho-Bernardo Study</td>
<td>Ratio of n-6 to n-3: Mean ± SD n-6 to n-3 PUFA: 7.9 ± 2.2, Mean ± SD LA to ALA: 8.4 ± 2.3</td>
<td>Ratio of n-6 to n-3: Inverse association with total hip (LA to ALA only) and LS BMD</td>
</tr>
<tr>
<td>Muraki, 2007 (190)</td>
<td>632 PM women, Japanese</td>
<td>Fish: ≤5 servings/wk vs. &gt;5 servings/wk</td>
<td>Fish: No difference in LS BMD</td>
</tr>
<tr>
<td>Zalloua, 2007 (191)</td>
<td>6207 women (32% PM), Rural Chinese</td>
<td>Fish: &gt;250 g seafood/wk</td>
<td>Fish: Increase hip and total body BMD</td>
</tr>
<tr>
<td>Rousseau, 2009 (192)</td>
<td>118 men and 129 women, Caucasian (98%) American</td>
<td>Total n-3: &gt;1.27 g/d (men and women)</td>
<td>Total n-3: Increase femur neck and total femur BMD (men and women)</td>
</tr>
<tr>
<td>Chen, 2010 (193)</td>
<td>685 PM women, Chinese</td>
<td>Fish: Quintile 1 (0.6 g/d) vs. Quintile 5 (64.7 g/d) from sea fish</td>
<td>Fish: Positive dose-dependent relationship with hip, LS, and whole body BMD and BMC, Decrease hip and total fracture risk</td>
</tr>
<tr>
<td>Nawata, 2013 (194)</td>
<td>205 PM women, Japan</td>
<td>Total n-3: Average: 2.7 g/d</td>
<td>Total n-3: Increase LS BMD</td>
</tr>
<tr>
<td>Mangano, 2014 (195)</td>
<td>2125 older men and women (48% women), multi-ethnic American, NHANES 2005-08</td>
<td>EPA+DHA: 0.15 g/d from supplements (men and women)</td>
<td>EPA+DHA: Increase LS BMD (men and women)</td>
</tr>
<tr>
<td>Harris, 2015 (196)</td>
<td>266 PM women, multi-ethnic American, BEST Study</td>
<td>Total n-6: Tertile 1 (&lt;8.51 g/d) vs. Tertile 3 (&gt;11.46 g/d) AA: Average: 0.11 ± 0.06 g/d Total n-3: Tertile 1 (&lt;0.99 g/d) vs. Tertile 3 (&gt;1.40 g/d) Ratio of n-6 to n-3: Mean ± SD n-6 to n-3 PUFA: 8.27 ± 1.74, Mean ± SD LA to ALA: 9.15 ± 2.04</td>
<td>Total n-6: Decrease total body BMD AA: Increase trochanter BMD Total n-3: Decrease total body BMD Ratio of n-6 to n-3: Decrease total body BMD</td>
</tr>
<tr>
<td>Prospective Studies</td>
<td>Fish: Quartile 1 (&lt;1 serving/mo) vs. Quartile 4 (&gt;1 serving/wk)</td>
<td>Fish: Decrease hip fracture risk</td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>---------------------------------------------------------------</td>
<td>----------------------------------</td>
<td></td>
</tr>
<tr>
<td>Feskanich, 2003 (197)</td>
<td>72,000 PM women, 18yr follow-up, multi-ethnic American, NHS</td>
<td>Total PUFA: Quartile 1 (0.71-5.16% energy) vs. Quartile 4 (7.90-31.84% energy)</td>
<td></td>
</tr>
<tr>
<td>Orchard, 2010 (198)</td>
<td>137,486 PM women, 7.8yr follow-up, multi-ethnic American, WHI</td>
<td>Total n-6: Quartile 1 (0.61 g/d) vs. Quartile 4 (6.94 g/d)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total n-3: Quartile 1 (0.08 g/d) vs. Quartile 4 (2.1 g/d)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALA: Quartile 1 (0.08 g/d) vs. Quartile 4 (0.84 g/d)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>EPA+DHA: Quartile 1 (0 g/d) vs. Quartile 4 (0.09 g/d)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ratio of n-6 to n-3: n-6 to n-3 PUFA &gt;6.43</td>
<td></td>
</tr>
<tr>
<td>Virtanen, 2010 (199)</td>
<td>2239 men and 2962 women, 11yr follow-up, multi-ethnic American, Cardiovascular Health Study</td>
<td>Total PUFA: Quarte 1 (7.9 g/d) vs. Quarte 5 (13.9 g/d)</td>
<td></td>
</tr>
<tr>
<td>Farina, 2011 (200)</td>
<td>530 PM women, 4yr follow-up, elderly Caucasian American, Framingham Osteoporosis Study</td>
<td>Total n-6: Quartile 1 (6.9 g/d) vs. Quarte 5 (12.4 g/d)</td>
<td></td>
</tr>
<tr>
<td>Virtanen, 2012 (201)</td>
<td>75,878 women free of osteoporosis at baseline, 24yr follow-up, multi-ethnic American, NHS</td>
<td>LA: Quarte 1 (6.8 g/d) vs. Quarte 5 (12.1 g/d)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total n-3: Quarte 1 (0.9 g/d) vs. Quarte 5 (1.6 g/d)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total PUFA: Decrease hip fracture risk</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Total n-6: Decrease hip fracture risk</td>
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<tr>
<td></td>
<td></td>
<td>LA: Decrease hip fracture risk</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total n-3: Decrease hip fracture risk</td>
<td></td>
</tr>
</tbody>
</table>
| Jarvinen, 2012 (202) | 554 PM women, 3yr follow-up, Finnish, Kuopio Fracture Prevention Study | **ALA:** Quintile 1 (0.7 g/d) vs. Quintile 5 (1.2 g/d)  
**EPA+DHA:** Quintile 1 (0.07 g/d) vs. Quintile 5 (0.37 g/d)  
**Fish:** Quintile 1 (<1 serving/month) vs. Quintile 5 (≥5 servings/week) | **ALA:** No change in fracture risk  
**EPA+DHA:** Decrease hip fracture risk  
**Fish:** Decrease hip fracture risk |
|----------------------|-----------------------------------------------------------------|-----------------------------------------------------------------|
| **Total PUFA:** | Quartile 1 (≤7.11 g/d) vs. Quartile 4 (>10.24 g/d)  
**Total n-6:** Quartile 1 (4.89 g/d) vs. Quartile 4 (6.89 g/d)  
**LA:** Quartile 1 (4.8 g/d) vs. Quartile 4 (6.77 g/d)  
**Total n-3:** Quartile 1 (1.31 g/d) vs. Quartile 4 (2.21 g/d)  
**ALA:** Quartile 1 (1.02 g/d) vs. Quartile 4 (1.73 g/d) | **Total PUFA:** Increase LS BMD  
**Total n-6:** Increase LS and total body BMD  
**LA:** Increase LS and total body BMD  
**Total n-3:** Increase total body BMD  
**ALA:** Increase LS BMD |

1 AA; arachidonic acid, ALA; alpha-linolenic acid, BEST; The Bone and Estrogen Strength Training Study, BMC; bone mineral content, BMD; bone mineral density, DHA; docosahexaenoic acid, EPA; eicosapentaenoic acid, LA; linoleic acid, LS; lumbar spine, NHANES; National Health and Nutrition Examination Survey, NHS; Nurses’ Health Study, PM; postmenopausal, PUFA; polyunsaturated fatty acid, WHI; Women’s Health Initiative

2 Associations lost after multivariate adjustment for age, race-ethnicity, education, marital status, family history of fracture, fracture after age 54yr, number of falls in past 12 months, height, weight, total vitamin D intake, calcium intake, hormone therapy use, antianxiety or antidepressant medication use, bisphosphonate use, corticosteroid use, smoking status, arthritis, depression, general health, parity, treated diabetes, and weekly exercise.

3 Associations lost after multivariate adjustment for age, body mass index, smoking status, physical activity, thiazide-type diuretic use, multivitamin use, osteoporosis, cardiovascular disease and cancer, intakes of total energy, calcium, protein, vitamin D, vitamin K, retinol, caffeine, alcohol, and postmenopausal hormone use.
Findings from Observational Studies

Fatty Acid Intake: LA, ALA, EPA, DHA, total PUFA, total n-6, and total n-3

Risk of Fracture:

Examination of the association between dietary intake of PUFAs and the risk of hip fracture has resulted in conflicting findings in several large observational studies. Analysis of the Nurse’s Health Study (NHS), in which postmenopausal women were followed for 24 years, showed a significant age-adjusted reduction in risk of hip fracture of 0.78 [95% CI 0.64, 0.94] among women in the highest quintile of total n-3 PUFA intake (1.6 g/day) compared to those in the lowest quintile (0.9 g/day) (201). EPA and DHA intake also showed a significant age-adjusted, hip fracture risk reduction of 0.88 [95% CI 0.72, 1.07] between women in the highest (0.37 g/day) and the lowest (0.07 g/day) quintile of intake, but after further multivariate-adjustment, all associations were lost. No associations with ALA, the major constituent of total n-3 PUFA in the diet, were observed with hip fracture risk. Inverse associations between LA intake and hip fracture risk were found among women in the highest (12.1 g/day) compared with the lowest (6.8 g/day) quintile of intake. The multivariate-adjusted relative risk was 0.81 [95% CI 0.67, 0.98] in the highest compared to the lowest quintile of LA intake among women. Although LA has long been considered a precursor of pro-inflammatory eicosanoids, which have a negative impact on bone metabolism, these results suggest a benefit to consuming more than 6.8 g/day, and possibly up to 12.1 g/day. While a significant reduction in risk was observed between the highest and lowest quintile of LA intake, a modest reduction in risk was evident in the second quintile, with little to no additional benefit with higher intake of LA (201). These findings support a minimum requirement
of more than 6.8 g/day of LA intake to provide a benefit to bone health in women, and that intake as high as 12.1 g/day does not cause a detriment.

In another large cohort of 137,486 postmenopausal American women enrolled in the Women’s Health Initiative (WHI), a higher intake of total PUFAs, supplied primarily from n-6 fatty acids, was associated with a lower total fracture risk after an average follow-up of 7.8 years (198). Age-adjusted total fracture rates decreased with increasing n-3 PUFA intake when stratified by quartiles and the lowest hip fracture rates were associated with the highest n-3 intake (2.1 g/day). ALA was the main n-3 PUFA in the diet, with women consuming an average of 1.27 g/day and an average EPA+DHA intake of 0.13 g/day. A significant reduction in total fracture risk was observed in women in the highest quartile of ALA consumption when adjusted for age and ethnicity but not with further adjustment. While women with the highest intake of EPA+DHA (0.17 g/day) experienced a lower risk of hip fracture in a model adjusted for age and ethnicity, an increased risk of total fractures was reported after multivariate adjustment. This multivariate model adjusted for factors including family history of fracture, previous fracture incidence, intake of bone-positive nutrients, exercise, parity, and smoking status. Because the intake of EPA+DHA was very low in this population (0.08 – 0.17 g/day), it is not possible to examine associations at higher PUFA intakes. Similar to associations observed with ALA intake, high consumption of n-6 PUFAs (6.94 g/day), predominately in the form of LA, was also associated with a decrease in total fracture risk compared to women in the lowest quartile (0.61 g/day) of n-6 PUFA consumption (198). Together, findings from these large prospective studies support a lower risk of fragility fracture with higher total n-6 PUFA intake (primarily from LA). No other associations remained
after multivariate adjustment. The large prospective studies adjusted for a multitude of covariates, including anthropometric measures (Table 2-7). Authors of the NHS study included the use of multiple medications and general health status as covariates (201). Authors of the WHI however, adjusted for the total intake of bone-supporting nutrients in their population as well as general health status. The independent effect of each variable on the association between n-3 PUFA (ALA or EPA and DHA) and risk fracture is unknown.

**BMD:**

In a 3-year prospective study of postmenopausal women from Finland, a significant increase in multivariate-adjusted lumbar spine BMD was observed with increasing intake of certain PUFA types (202). From the lowest to highest quartiles of intake of total PUFA, LA, ALA, and total n-6 fatty acid, an increase in BMD of the lumbar spine was observed (202). A similar significant pattern was observed for total body BMD with increasing LA, total n-6 fatty acid, and total n-3 fatty acid intake. No associations were found for EPA+DHA, or AA intake with lumbar spine or total body BMD. Similarly, in a pooled population of older men and women, above average total n-3 fatty acid intake (>1.27 g/day) was associated with greater femur neck BMD and total femur BMD (192). No associations were observed with ALA, EPA, DHA, or EPA+DHA intake in women, suggesting that an overall increase in total n-3 PUFA is important for bone health in this population.

In a cross-sectional study of postmenopausal Japanese women, total n-3 fatty acid intake, where the primary source of n-3 PUFA was from fish, was positively associated with lumbar spine BMD (194). The average daily n-3 PUFA intake was 2.7 g/day and
these findings were independent of bone resorption and formation markers. Results from a secondary analysis of 266 postmenopausal women enrolled in the Bone Estrogen Strength Training (BEST) Study found a positive association between AA intake (0.11 ± 0.06 g/day) and trochanter BMD in women not receiving hormone replacement therapy (196). A significant negative association in total body BMD was observed with increasing tertiles of n-6 and n-3 fatty acid intake (196).

Most recently, the relation of n-3 PUFA supplement use and BMD was examined in a diverse dataset of the US population using the NHANES (2005-08) (195). In a population of 2125 men and women over 60, use of n-3 fatty acid supplements showed a positive association with lumbar spine BMD, but not femur neck or total femur BMD using DXA. Within this study, only 15% of individuals reported using n-3 fatty acid supplements (195). In summary, consistent positive associations between total PUFA, LA, total n-6, and total n-3 and site-specific BMD have been observed in several observational (prospective and cross-sectional) studies. Less consistent findings support the positive association between the n-6, AA and the n-3 PUFA, ALA.

**Ratio of Fatty Acid Intake**

As discussed earlier, LA and ALA and their longer chain derivatives share common enzymes for elongation and desaturation however, the metabolically active products have roles as pro- and anti-inflammatory mediators in the body (Figure 2-4). These two contrasting roles of pro- and anti-inflammatory mediators suggest the importance of the PUFA ratio in the diet.

*Risk of Fracture:*
The NHS and WHI considered the PUFA ratio in their analyses. *Results from these two large cohorts show no significant relationships associated with the dietary ratio of total n-6 to n-3 fatty acids and hip fracture risk.* In the NHS, the ratio varied from less than 6.7 in the lowest quintile to greater than 8.9 in the highest (201). This range may have been too narrow to detect any differences in fragility fracture risk in the population. From the WHI, dietary PUFA ratios spanned from 0.49 to 39.01 with an average ratio of 7.6 (198). A 7% reduction in relative risk of total fractures was discovered in this population of postmenopausal women when the total n-6 to n-3 PUFA ratio was above 6.4 compared to those with a PUFA intake ratio below 6.4. This data provides support for the importance of attaining a minimum daily dietary intake of n-6 PUFA for the proper maintenance of bone health.

*BMD:*

In a fully adjusted model of analysis of postmenopausal women (not using hormone replacement therapy) enrolled in the Rancho-Bernardo study, the ratio of total n-6 to total n-3 PUFA was negatively associated with total hip and lumbar spine BMD (189). This population of mid-upper class, white residents of Southern California consumed a ratio of total n-6 to n-3 fatty acids (7.9 ± 2.2) made up of 10.2 g/day and 1.3 g/day n-6 and n-3 PUFAs, respectively. In contrast, findings from women enrolled in the BEST Study (196) or those in the Framingham Osteoporosis Study (200) showed no associations between PUFA ratio (average of BEST: 8.27 ±1.74, average of Framingham Osteoporosis Study: 8.22 ± 2.89) and BMD. The dietary PUFA ratio in these studies are relatively low compared to the national intake in the USA, estimated between 15 through 17:1 (155).
**Fish Consumption**

Although intake of individual fatty acids and their association with fracture provide us with specific information regarding the benefits of PUFA consumption on fracture risk, it is important to also consider the association with a food source, such as fish. Fatty fish are rich sources of EPA and DHA, although the levels of each vary widely among fish species. Moreover, fatty fish (i.e., swordfish, salmon, mackerel, sardines, salmon) are also a good source of vitamin D and protein, critical for bone health.

*Risk of Fracture:*

In the 18-year follow-up study of the NHS, consisting of over 72,000 postmenopausal women, consumption of a 85-142 g serving of fatty fish more than once per week, resulted in a 33% lower risk of hip fracture compared to those who consumed fatty fish less than once per month (197). Separate analysis of the same population of white American women, followed for 24 rather than 18 years, showed a significant reduction in age-adjusted hip fracture risk among women who consumed more than 5 servings per week of fish compared to those who consumed less than 1 serving per month (201).

*BMD:*

The observational studies that used BMD as a surrogate measure of risk of fracture to investigate the association between fish and bone health have provided conflicting results. A study of 632 postmenopausal Japanese women found no significant difference in lumbar spine BMD between women who consumed fish more than 5 times per week compared to those who consumed fish less than 5 times per week (190).
authors of this study did not clarify the type of fish consumed, but do suggest that both

groups may have exceeded the intake level necessary to provide any further benefit.

Another large cohort of 5201 men and women found a negative effect of more frequent
intake of tuna and other fish, citing a slightly lower average femur neck BMD between
the highest (≥ 3 servings per week, corresponding to 0.59 g/day EPA+DHA) and lowest
(< 1 serving per month, 0.05 g/day EPA+DHA) quintiles of intake where average intake
was 1.6 servings per week (199). There was no association between EPA+DHA intake
and risk of fracture in this population. In this study of older (average age 72.8 years),
community-dwelling participants, although more frequent fish intake was negatively
associated with femur neck BMD these associations were not paralleled with an increased
fracture incidence when stratified by intake of EPA+DHA (199).

In direct contrast, three prospective studies - two cohorts of Chinese populations
and the Framingham Osteoporosis cohort - reported a positive association of higher fish
consumption on BMD (191, 193, 200). An intake of 250 g/week or greater (roughly 3
servings per week) of seafood, including fish and shellfish, was associated with a greater
total body and hip BMD in rural Chinese postmenopausal women (191). Similarly, a
dose-dependent linear relation was observed between intakes of sea fish (non freshwater
or shellfish) and BMD or BMC at the whole body, total hip, femur neck, trochanter, and
intertrochanter in 685 postmenopausal women (193). This association corresponded to a
significantly lower odds ratio of osteoporosis at the whole body, total hip, femur neck,
and intertrochanter for women consuming the highest (64.7 g/day) compared to those in
the lowest (0.6 g/day) quintile of intake of sea fish (193). In the Framingham
Osteoporosis Study, consumption of ≥ 3 servings of fish per week was associated with
the maintenance of femur neck BMD in older women over a 4-year follow-up period (200). This association can be mainly attributed to fatty fish consumption, as women with high intakes of fatty fish lost significantly less femur neck BMD than did women with moderate or low intakes. These associations were also observed with total fish intake in women, but were not significant. In the Framingham Osteoporosis Study, the type of fish consumed was mainly tuna and white and other fish, while fatty fish accounted for 12% of intake (200).

The conflicting results in these studies of fish consumption and BMD may be attributed to the type of fish consumed. In the observational studies citing no benefit with fish intake, the sub-type of fish was either not classified (190) or was classified as either tuna or broiled or baked fish, or fried fish (199). Benefits were observed in those studies quantifying fish and shellfish (191), sea fish (193), or total fish, fatty fish + tuna, fatty fish, or tuna intakes (200). The positive effects of fish consumption in these studies may be attributed to their displacement of low-quality food items from the diet, the high vitamin D levels, and/or the high content of long chain n-3 PUFAs found within these types of fish. The total PUFA, n-6 PUFA, and n-3 PUFA content of different fish species varies substantially. Table 2-8 summarizes the total fat and PUFA contents of a variety of fish species and has been adapted from data published by Strobel and colleagues (203).

In summary, findings from observational studies reveal higher intakes of total PUFA, total n-6, LA, total n-3 and fatty fish are associated with higher BMD and a lower risk of fragility fracture in women. Less consistent benefits to bone health are associated with higher intake of long chain n-3 PUFAs or when the dietary ratio of n-6 to n-3 PUFA is considered. When comparing findings across observational studies, it is important to
consider many aspects of the study including the population characteristics, current dietary patterns, and primary outcome measures (Table 2-9).
Table 2-8. Total fat, n-6, n-3, and EPA+DHA contents of various fish species

<table>
<thead>
<tr>
<th>Fish Species</th>
<th>Total Fat</th>
<th>Total n-6</th>
<th>Total n-3</th>
<th>EPA+DHA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fatty Fish</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Salmon, farmed</td>
<td>12.3</td>
<td>1.32</td>
<td>2.40</td>
<td>1.53</td>
</tr>
<tr>
<td>Salmon, wild</td>
<td>2.07</td>
<td>0.05</td>
<td>0.53</td>
<td>0.44</td>
</tr>
<tr>
<td>Herring</td>
<td>12.7</td>
<td>0.31</td>
<td>1.95</td>
<td>1.45</td>
</tr>
<tr>
<td>Mackerel</td>
<td>20.6</td>
<td>0.59</td>
<td>4.64</td>
<td>3.08</td>
</tr>
<tr>
<td>Anchovies, drained of oil</td>
<td>6.21</td>
<td>2.31</td>
<td>0.93</td>
<td>0.66</td>
</tr>
<tr>
<td>Sardine, drained of oil</td>
<td>10.2</td>
<td>2.45</td>
<td>1.40</td>
<td>1.16</td>
</tr>
<tr>
<td><strong>Non-Fatty Fish</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tilapia</td>
<td>1.63</td>
<td>0.29</td>
<td>0.12</td>
<td>0.06</td>
</tr>
<tr>
<td>Tuna</td>
<td>2.28</td>
<td>0.14</td>
<td>0.78</td>
<td>0.72</td>
</tr>
<tr>
<td>Perch, fillet</td>
<td>0.80</td>
<td>0.05</td>
<td>0.12</td>
<td>0.08</td>
</tr>
<tr>
<td>Prawn</td>
<td>0.53</td>
<td>0.09</td>
<td>0.11</td>
<td>0.10</td>
</tr>
<tr>
<td>Alaskan pollock, fillet</td>
<td>0.77</td>
<td>0.02</td>
<td>0.39</td>
<td>0.37</td>
</tr>
<tr>
<td>Alaskan pollock, breadcrued</td>
<td>7.85</td>
<td>4.04</td>
<td>0.30</td>
<td>0.16</td>
</tr>
</tbody>
</table>

1 Data from (203)

2 The amount of fat is expressed as a percentage of fresh fish weight (g/100g).
Table 2-9. Aspects to consider when reviewing observational studies assessing the effect of PUFA intake on bone health

<table>
<thead>
<tr>
<th>Participant Baseline Characteristics</th>
<th>Dietary Patterns</th>
<th>Primary Outcome Measure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, sex, ethnicity, body mass index, physical activity, general health status, years since menopause, hormone therapy use, previous fracture incidence, medication use, protein intake, vitamin D intake, and calcium intake are all variables that modulate bone health.</td>
<td>FFQ: captures habitual dietary patterns; generally less detailed than other methods; more likely to overestimate intake; less expensive. 24-h Recall, food diaries: capture a snapshot of dietary patterns; time consuming. Dietary patterns and supplement use: may change over time with changing public health recommendations. Accessibility to fresh food items: changes with season, geographical region, and economic burden. Range of intake: variation of dietary intake among the sample population must be wide enough to establish distinct subgroups of intake. Follow-up time: wide range of average follow-up times among studies (3 to 24yrs). Ability of the diet assessment tool to accurately estimate intake of fatty acid chain length and saturation index, i.e., n-3 PUFAs from plant or marine sources. Sensitivity of the diet assessment tool to differentiation between fatty acid sources, i.e., fatty fish vs. fried fish vs. canned fish, etc.</td>
<td>Fracture risk: clinically relevant measure; results may be masked by a short follow-up period, error in self-reporting fractures, or changes to fall incidence with dietary patterns. BMD by DXA: measures skeletal site-specific effects; changes may be observed without concomitant changes in fracture incidence. High-resolution pQCT: nondestructive imaging techniques that allow for the 3D quantification of bone structure; not used in any human studies reviewed in this thesis.</td>
</tr>
</tbody>
</table>

1 BMD; bone mineral density, DXA; dual energy X-ray absorptiometry, FFQ; food frequency questionnaire, pQCT; peripheral quantitative computed tomography, PUFA; polyunsaturated fatty acid.
Findings from RCTs

While observational studies of the population identify associations between dietary habits and bone health, they do not allow for causal relationships to be investigated. RCTs have been conducted to determine whether a cause and effect relationship exists between dietary PUFA intake and markers of bone health in postmenopausal women. Most studies have shown either a benefit or a null effect of PUFA supplementation to bone health (Table 2-10).
Table 2-10. Summary of findings from RCTs concerning PUFA intake and bone health

<table>
<thead>
<tr>
<th>Reference</th>
<th>Population Characteristics</th>
<th>Intervention</th>
<th>Duration</th>
<th>Bone-Related Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Studies of Flaxseed or ALA</strong></td>
<td></td>
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<tr>
<td>Lucas, 2002 (204)</td>
<td>n=36 PM women</td>
<td>40 g/d wheat germ, 40 g/d whole flaxseed</td>
<td>12 weeks</td>
<td>• No effect of supplementation on serum or urinary markers of bone metabolism</td>
</tr>
<tr>
<td>Brooks, 2004 (205)</td>
<td>n=46 PM women</td>
<td>Placebo, 25 g/d whole flaxseed, 25 g/d soy flour</td>
<td>16 weeks</td>
<td>• No effect of supplementation on serum or urinary markers of bone metabolism</td>
</tr>
<tr>
<td>Dodin, 2005 (206)</td>
<td>n=179 PM women</td>
<td>40 g/d wheat germ, 40 g/d whole flaxseed</td>
<td>12 months</td>
<td>• No effect of supplementation on lumbar spine or femur neck BMD</td>
</tr>
<tr>
<td>Griel, 2007 (207)</td>
<td>n=23 men and women (n=3 PM women)</td>
<td>LA: ALA (9.5:1), LA: ALA (3.5:1), LA: ALA (1.6:1)</td>
<td>6 weeks, 3 week wash-out</td>
<td>• Lower PUFA ratio decreased serum NTX and TNFα concentrations</td>
</tr>
<tr>
<td>Cornish, 2009 (208)</td>
<td>n=23 PM women</td>
<td>30 mL/d corn oil, 30 mL/d flaxseed oil (both undergoing resistance exercise)</td>
<td>12 weeks</td>
<td>• No effect of supplementation on lumbar spine or hip BMD</td>
</tr>
<tr>
<td><strong>Studies of Fish oil, EPA, DHA or EPA+DHA</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Vanpapendorp, 1995 (209)</td>
<td>n=40 women with confirmed osteoporosis</td>
<td>4 g/d Olive Oil, 4 g/d EPO, 4 g/d Fish oil, 4 g/d EPO + Fish oil</td>
<td>16 weeks</td>
<td>• Fish oil supplementation increased serum osteocalcin compared to EPO • Olive oil, fish oil, EPO + Fish oil increased procollagen concentrations from baseline</td>
</tr>
<tr>
<td>Kruger, 1998 (210)</td>
<td>n=60 PM women with confirmed osteoporosis</td>
<td>6 g/d coconut oil (97% SFA), 6 g/d EPO + fish oil (60% LA, 8% GLA, 4% EPA, 3% DHA</td>
<td>18 months (phase I) + 18 months (phase II)</td>
<td>• Both interventions increased procollagen, BALP, osteocalcin, and deoxyypyridinoline • EPO + fish oil supplementation preserved lumbar spine and femur neck BMD in phase I and phase II</td>
</tr>
<tr>
<td>Study</td>
<td>Participants</td>
<td>Intervention</td>
<td>Duration</td>
<td>Findings</td>
</tr>
<tr>
<td>-------------------------------</td>
<td>--------------</td>
<td>------------------------------------------------------------------------------</td>
<td>----------</td>
<td>--------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Bassey, 2000 (211)</td>
<td>n=45 PM women</td>
<td>1 g/d calcium, 1 g/d calcium + 4 g/d EPO + 440 mg/d fish oil</td>
<td>12 months</td>
<td>• No effect of supplementation on total body BMD or urinary markers of bone metabolism</td>
</tr>
<tr>
<td>Appleton, 2011 (212)</td>
<td>n=190 men and women</td>
<td>1.48 g/d Olive oil, 0.63 g/d EPA + 0.85 g/d DHA</td>
<td>12 weeks</td>
<td>• No effect of supplementation on serum CTX concentrations</td>
</tr>
</tbody>
</table>
| Hutchins-Weise, 2014 (213)    | n=34 women on aromatase inhibitors | 4 g/d Safflower oil 2.52 g/d EPA + 1.68 g/d DHA | 12 weeks | • EPA+DHA supplementation decreased serum CTX compared to control  
• Serum CTX concentrations were positively correlated with serum AA and with serum n-6 to n-3 ratio and negatively correlated with serum EPA+DHA concentrations |

1 AA; arachidonic acid, BALP; bone specific alkaline phosphatase, BMD; bone mineral density, CTX; C-terminal telopeptide, DHA; docosahexaenoic acid, EPA; eicosapentaenoic acid, EPO; evening primrose oil, GLA; γ-linoleic acid, LA; linoleic acid, NTX; N-terminal telopeptide, PM; postmenopausal, PUFA; polyunsaturated fatty acid, SFA; saturated fatty acid, TNFα; tumor necrosis factor alpha.
One of the first trials investigated the effects of evening primrose oil, a natural source of the n-6 PUFA γ-linoleic acid, fish oil, or the combination of oils on serum biochemical markers of elderly women with osteoporosis (209). In this study, 4 g/day of fish oil for 16 weeks showed a beneficial effect via an increase in serum osteocalcin and procollagen concentrations, biochemical markers of bone turnover and formation respectively (209). More recent support of long chain n-3 PUFAs on bone health comes from a study investigating the effect of 2.52 g EPA and 1.68 g DHA/day compared to 4 g safflower oil control/day in postmenopausal women receiving aromatase inhibitors for the treatment of estrogen-positive breast cancer (213). Following 12 weeks of treatment, women in the EPA+DHA supplementation group experienced a significant decrease in serum C-terminal telopeptide (CTX), and this marker of bone resorption was positively correlated with serum AA and with serum long chain n-6 to n-3 fatty acid ratios and negatively correlated with serum EPA+DHA concentrations (213). Of importance to note, all women received a 1000 mg calcium supplement and 800 IU vitamin D/day and not all women experienced a similar response, suggesting that these women were ‘non-responders’ and were retroactively removed from the analyses. This study did not evaluate baseline serum calcium and vitamin D concentrations, but it is possible that supplementation of all women upon entrance into the study may have masked some of the effects of EPA+DHA supplementation.

Conversely, ancillary analysis of a large RCT consisting of 190 men and women consuming either 1.48 g EPA+DHA/day or olive oil placebo found no change in CTX (212). Although average fasting plasma concentrations of n-3 PUFAs increased over the 12-week study, no association was found with this marker of bone resorption. This
finding could be due to the variability in participant characteristics, which included both men and women of a wide age-range, of which only 75% were over 50 years and most likely not experiencing menopause.

There is insufficient evidence to suggest a positive effect of ALA on postmenopausal bone health. In one study of 179 women with at least 6 months of amenorrhea, neither 40 g of flaxseed (roughly 9.2 g ALA/day) or wheat germ placebo incorporated into the diet prevented the progressive bone loss observed over 12 months in early menopausal women (206). Although this study saw no change in lumbar spine or femur neck BMD by DXA between the two groups, this could be attributed to the early stage of menopause women were classified in and the overall good health status of the participants, inferred by the recruitment criteria of the study. Bone loss at the hip and lumbar spine over the menopausal transition is most dramatic during late peri-menopause (3 to 11 months amenorrhea) and post-menopause (> 12 months amenorrhea) (214), which coincides with the relatively early stage of the menopausal transition women from this study were classified in (206). Similarly, no change in lumbar or hip BMD was observed after 12 weeks of resistance training with and without the supplementation of 30 mL flaxseed oil/day (approximately 14 g ALA/day) (208). The design of this trial could not distinguish the effect of diet alone on bone health as all 51 Canadian men and women, 23 of whom were postmenopausal females, underwent resistance training. Flaxseed supplementation trials of 40 g/day of whole ground flaxseed for 3 months (roughly 9.2 g ALA/day) (204) and 25 g/day for 4 months (roughly 5.8 g ALA/day) (205) in postmenopausal women resulted in no effect on biomarkers of bone metabolism when compared to their wheat placebo or wheat and soy supplementation controls, respectively.
To establish the long-term effect of a complex combination of PUFAs in an elderly population of women with osteoporosis, supplementation with 6 g evening primrose oil and fish oil was compared with 6 g of coconut oil/day over 18 months (210). The PUFA supplement consisted of approximately 68% n-6 fatty acid and 7% long chain n-3 fatty acid, while the coconut oil treatment was rich in saturated fatty acids. Both interventions resulted in significant increases in markers of bone formation (procollagen, bone alkaline phosphatase), and decreases in markers of bone turnover (osteocalcin) and resorption (deoxypyridinoline). The similar findings observed with both treatments may be attributed to the calcium supplement (600 mg) provided to all women enrolled in the study. Upon entrance into the study, the average daily calcium intake was 653 ± 249 mg, below the 1998 recommended dietary allowance of 800 mg and the current dietary recommended intake (US, Canada) of 1200 mg for women over 70 years of age. Thus, their bone health likely benefited from this calcium supplementation, rather than the fatty acid intervention. Interestingly, PUFA supplementation with evening primrose and fish oil was able to preserve lumbar spine BMD and increase femur neck BMD over the 18-month treatment period, while coconut oil caused a 3.2% and 2.1% decrease in lumbar spine and femur neck BMD, respectively. Because of the beneficial effects observed with PUFA supplementation, 21 women remained in the study for a total of 36 months, and those in the coconut oil group were reallocated to receive evening primrose and fish oil. After 36 months, lumbar spine and femur neck BMD was preserved in those women who had consistently been supplemented with the PUFA combination and was increased in the women switched to the PUFA supplement (210). In contrast, a study of 45 postmenopausal women consuming either 1 g calcium with 4 g evening primrose and 440
mg fish oil, or calcium alone found a decrease in total body BMD by 1% in both groups over 12 months and no between group differences in urinary markers of bone turnover (211). The women recruited in this study already had an average baseline calcium intake of approximately 900 mg/day, which may have masked any effect attributed to the fatty acid supplementation.

To our knowledge, the only randomized crossover control trial to assess the effect of the ratio of n-6 to n-3 PUFAs on bone health comes as ancillary data from a larger study designed to evaluate the effect of PUFAs in the diet on cardiovascular disease (207). Three diets were used, lasting a period of 6 weeks and separated by a 3-week washout period. All diets consisted of 35% fat as energy based on 2400 kcal/day and differed by PUFA ratio. As a control, created to mimic the average American diet, a ratio of 9.5 for LA to ALA was consumed. Two experimental diets consisted of ratios of 3.5 and 1.6, respectively. At study endpoint, the lowest ratio of dietary n-6 to n-3 yielded a significantly lower serum N-terminal telopeptide (NTX) concentration. This treatment diet also reduced serum concentrations of the inflammatory marker, tumor necrosis factor alpha (TNFα), and a positive correlation was observed between NTX levels and TNFα concentrations across all diets. Although bone resorption rates seem to be positively affected by PUFA ratio, serum bone alkaline phosphatase levels were not changed with this diet. Although the crossover design is powerful, this study included 23 participants, of which only 3 were postmenopausal women and the remainder were men, and all were at high risk for cardiovascular disease. These results suggest the need for more controlled feeding studies with crossover design, particularly studies including postmenopausal women, to evaluate how the level and ratio of n-6 to n-3 PUFAs modulates bone health.
In summary, findings from RCTs have suggested some benefit to bone health with supplementation with fish oil (with or without evening primrose oil) and the long chain n-3 PUFAs, EPA and DHA in the diet (209, 210, 213) but a null effect of the plant source, ALA (204-206, 208). When reviewing RCTs assessing the effect of PUFA intake on bone health, aspects to consider include participant inclusion criteria, baseline dietary patterns, intervention type and length, and the primary outcome measure (Table 2-11).
Table 2-11. Aspects to Consider When Reviewing RCTs Assessing the Effect of PUFA Intake and Bone Health

<table>
<thead>
<tr>
<th>Inclusion Criteria</th>
<th>Participant characteristics: age, sex, ethnicity, body mass index, physical activity, general health status, and previous fracture incidence are all variables that modulate bone health</th>
</tr>
</thead>
</table>
| Baseline Dietary Patterns | Habitual PUFA intake and supplement use: lifelong exposure to certain PUFAs may impact the ability of an intervention to alter bone health above a threshold  
Baseline calcium and vitamin D intake: most RCTs provide all groups with calcium and vitamin D supplements, which may mask any additional effect of an intervention if baseline values are lower than recommended |
| Intervention | PUFA intervention: PUFA type and source (food or supplement), PUFA amount, PUFA ratio |
| Primary Outcome Measure | Imaging technique used: BMD by DXA, 3-D quantification of bone structure by high-resolution pQCT  
Site-specific effects: regions of the skeleton may not respond uniformly to an intervention (e.g., hip, lumbar spine, appendicular limbs)  
Biochemical markers of bone turnover: circadian variation of markers, sampling in a fed or fasted-state, intra-individual variability. To reduce variability, it is useful to measure multiple markers as well as the longitudinal change in markers over time |

1 BMD; bone mineral density, DXA; dual energy X-ray absorptiometry, pQCT; peripheral quantitative computed tomography, PUFA; polyunsaturated fatty acid, RCT; randomized controlled trial
**Summary of Findings in Human Studies**

Findings from human studies largely show that a greater intake of total PUFA, total n-6 PUFA, total n-3 PUFA, and fish is associated with higher BMD or a lower risk of fragility fracture in adult women (*Table 2-12*). Less consistent benefits to bone health are associated with higher intake of long chain n-3 PUFA or when the dietary ratio of n-6 to n-3 PUFA is considered. The strongest evidence for benefits to bone is from studies of fish intake. Regular consumption of fish and seafood not only provides high quantities of PUFA but can also be rich sources of protein, vitamin D, calcium, and other vitamins and minerals, all of which are necessary for the maintenance of strong, fracture-resistant bones.
Table 2-12. Summary of the findings concerning PUFA intakes and bone-related outcomes in humans

<table>
<thead>
<tr>
<th>PUFA</th>
<th>DRI(^1)</th>
<th>Quantity</th>
<th>Bone-Related Outcome</th>
<th>Study Type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total PUFA</td>
<td>12.1 g/d</td>
<td>Quartile 1 (0.71-5.16% energy) vs. Quartile 4 (7.90-31.84% energy)</td>
<td>• Decrease total fracture risk</td>
<td>P</td>
<td>Orchard 2010 (198)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quintile 1 (7.9 g/d) vs. Quartile 5 (13.9 g/d)</td>
<td>• Decrease hip fracture risk</td>
<td>P</td>
<td>Virtanen 2012 (201)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quartile 1 (≤7.11 g/d) vs. Quartile 4 (&gt;10.24 g/d)</td>
<td>• Increase lumbar spine BMD(^2)</td>
<td>P</td>
<td>Jarvinen 2012 (202)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11.26 g/d</td>
<td>• No association with lumbar spine and total body BMD(^2)</td>
<td>CS</td>
<td>Harris 2015 (196)</td>
</tr>
<tr>
<td>Total n-6 PUFA</td>
<td>11 g/d</td>
<td>Quartile 1 (0.61 g/d) vs. Quartile 4 (6.94 g/d)</td>
<td>• Decrease total fracture risk</td>
<td>P</td>
<td>Orchard 2010 (198)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quintile 1 (6.9 g/d) vs. Quartile 5 (12.4 g/d)</td>
<td>• Decrease hip fracture risk</td>
<td>P</td>
<td>Virtanen 2012 (201)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quartile 1 (4.89 g/d) vs. Quartile 4 (6.89 g/d)</td>
<td>• Increase lumbar spine and total body BMD(^2)</td>
<td>P</td>
<td>Jarvinen 2012 (202)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Tertile 1 (&lt;8.51 g/d) vs. Tertile 3 (&gt;11.46 g/d)</td>
<td>• Decrease total body BMD(^2)</td>
<td>CS</td>
<td>Harris 2015 (196)</td>
</tr>
<tr>
<td>LA</td>
<td>11 g/d</td>
<td>Quintile 1 (6.8 g/d) vs. Quartile 5 (12.1 g/d)</td>
<td>• Decrease hip fracture risk</td>
<td>P</td>
<td>Virtanen 2012 (201)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quartile 1 (4.8 g/d) vs. Quartile 4 (6.77 g/d)</td>
<td>• Increase lumbar spine and total body BMD(^2)</td>
<td>P</td>
<td>Jarvinen 2012 (202)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>9.78 g/d</td>
<td>• No association with lumbar spine and total body BMD(^2)</td>
<td>CS</td>
<td>Harris 2015 (196)</td>
</tr>
<tr>
<td>Total n-3 PUFA</td>
<td>1.1 g/d</td>
<td>&gt;1.27 g/d</td>
<td>• Increase femur neck and total femur BMD</td>
<td>CS</td>
<td>Rousseau 2009 (192)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Quintile 1 (0.9 g/d) vs. Quartile 5</td>
<td>• Decrease hip fracture risk</td>
<td>P</td>
<td>Virtanen</td>
</tr>
</tbody>
</table>
### ALA

<table>
<thead>
<tr>
<th>1.1 g/d</th>
<th>40 g whole flaxseed/d (12 wk)</th>
<th>• No change in serum or urinary markers of bone metabolism</th>
<th>RCT</th>
<th>Lucas 2002 (204)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>25 g whole flaxseed/d (16 wk)</td>
<td>• No change in serum or urinary markers of bone metabolism</td>
<td>RCT</td>
<td>Brooks 2004 (205)</td>
</tr>
<tr>
<td></td>
<td>40 g flaxseed/d (12 mo)</td>
<td>• No benefit to bone loss at lumbar spine or femur neck</td>
<td>RCT</td>
<td>Dodin 2005 (206)</td>
</tr>
<tr>
<td></td>
<td>30 mL flaxseed oil/d (12 wk)</td>
<td>• No change in lumbar spine or hip BMD</td>
<td>RCT</td>
<td>Cornish 2009 (208)</td>
</tr>
<tr>
<td></td>
<td>Quartile 1 (0.08 g/d) vs. Quartile 4 (0.84 g/d)</td>
<td>• Decrease total fracture risk</td>
<td>P</td>
<td>Orchard 2010 (198)</td>
</tr>
<tr>
<td></td>
<td>Quintile 1 (0.7 g/d) vs. Quintile 5 (1.2 g/d)</td>
<td>• No change in fracture risk</td>
<td>P</td>
<td>Virtanen 2012 (201)</td>
</tr>
<tr>
<td></td>
<td>Quartile 1 (1.02 g/d) vs. Quartile 4 (1.73 g/d)</td>
<td>• Increase lumbar spine BMD&lt;sup&gt;2&lt;/sup&gt;</td>
<td>P</td>
<td>Jarvenin 2012 (202)</td>
</tr>
<tr>
<td>1.11 g/d</td>
<td>• No association with LS and total body BMD&lt;sup&gt;2&lt;/sup&gt;</td>
<td>CS</td>
<td>Harris 2015 (196)</td>
<td></td>
</tr>
</tbody>
</table>

### EPA+ DHA

| Up to 10% of AMDR consumed as EPA and/or DHA | 4 g fish oil/d compared with 4 g EPO/d (16 wk) | • Increase levels of serum osteocalcin and procollagen  
• Decrease levels of serum BALP | RCT | Van Papendorp 1995 (209) |
|---------------------------------------------|-----------------------------------------------|----------------------------------------------------------|-----|-----------------|
|                                             | Quintile 1 (0.145 g/d) vs. Quintile 5 (0.519 g/d) from fish | • No effect on BMD  
• No effect on hip fracture risk | P | Virtanen 2010 (199) |
|                                             | Quartile 1 (0 g/d) vs. Quartile 4 | • Decrease hip fracture risk | P | Orchard |
| Ratio of n-6 to n-3 PUFA | 10 | 6 g EPO + fish oil combination/d (18 mo) | • Increase levels of serum procollagen and BALP  
• Decrease levels of serum osteocalcin and urinary deoxypyridinoline  
• Preservation of lumbar spine BMD  
• Increase femur neck BMD | RCT | Kruger 1998 (210) |
<table>
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<th></th>
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<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>4 g EPO + 0.44 g fish oil/d (12 mo)</td>
<td>• No benefit to total body BMD loss</td>
<td>RCT</td>
<td>Bassey 2000 (211)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Mean ratio of n-6 to n-3 PUFA: 7.9  
Mean ratio of LA to ALA: 8.4 | • Inverse association with total hip (LA to ALA only) and lumbar spine BMD² | CS | Weiss 2005 (189) |
| Ratio of LA to ALA: 9.5 vs. 3.5 vs. 1.6 (6 wk) | • Decrease levels of NTX and TNFα | RCT | Griel 2007 (207) |
| Ratio of n-6 to n-3 PUFA > 6.43 | • Decrease total fracture risk | P | Orchard 2010 (198) |
| Greater AA intake when EPA+DHA intake is above 0.14 g/d | • Increase femur neck BMD | P | Farina 2011 (200) |
| Ratio of LA to ALA: 3.61 to >5.13 | • Inverse association (NS) with lumbar spine BMD² | P | Jarvenin 2012 (202) |
| (0.09 g/d) | • Increase total fracture risk³ | 2010 (198) |
| 1.0 g/d (24 wk) | • Decrease levels of TNFα and IL-6 | RCT | Tartiban 2010 (215) |
| 1.48 g/d (12 wk) | • No change in levels of CTX | RCT | Appleton 2011 (212) |
| Quintile 1 (0.07 g/d) vs. Quintile 5 (0.37 g/d) | • Decrease hip fracture risk³ | P | Virtanen 2012 (201) |
| 0.15 g/d from supplements | • Increase lumbar spine BMD | CS | Mangano 2014 (195) |
| 0.18 g/d | • No association with lumbar spine and total BMD² | CS | Harris 2015 (196) |
Mean ratio of n-6 to n-3 PUFA: 8.27
Mean ratio of LA to ALA: 9.15

<table>
<thead>
<tr>
<th>Fish</th>
<th>Two servings of fish/wk</th>
<th>3-4 servings/wk</th>
<th>• Decrease hip fracture risk</th>
<th>CC</th>
<th>Suzuki 1997 (216)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Quartile 1 (&lt;1 serving/mo) vs. Quartile 4 (&gt;1 serving/wk)</td>
<td>• Decrease hip fracture risk</td>
<td>P</td>
<td>Feskanich 2003 (197)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>≤5 servings/wk vs. &gt;5 servings/wk</td>
<td>• No difference in lumbar spine BMD</td>
<td>CS</td>
<td>Muraki 2007 (190)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;250 g seafood/wk</td>
<td>• Increase hip and total body BMD</td>
<td>CS</td>
<td>Zalloua 2007 (191)</td>
</tr>
<tr>
<td></td>
<td>Quintile 1 (0.6 g/d) vs. Quintile 5 (64.7 g/d) from sea fish</td>
<td>• Positive dose-dependent relationship with hip, spine, and whole body BMD and BMC • Decrease hip and total fracture risk</td>
<td>CS</td>
<td>Chen 2010 (193)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Quartile 1 (&lt;1 serving/mo) vs. Quartile 4 (≥3 serving/wk)</td>
<td>• Decrease femur neck BMD</td>
<td>P</td>
<td>Virtanen 2010 (199)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥3 servings/wk</td>
<td>• Maintenance of femur neck BMD</td>
<td>P</td>
<td>Farina 2011 (200)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Quintile 1 (&lt;1 serving/mo) vs. Quintile 5 (≥5 servings/wk)</td>
<td>• Decrease hip fracture risk³</td>
<td>P</td>
<td>Virtanen 2012 (201)</td>
<td></td>
</tr>
</tbody>
</table>

1 DRI, Females 51-70 y (154).

2 Women not receiving HRT only.

3 Significant associations were lost after full multivariate adjustment of the model.

4 ALA; alpha-linolenic acid, AMDR; acceptable macronutrient distribution range, BALP; bone specific alkaline phosphatase, BMC; bone mineral content, BMD; bone mineral density, CC; case-control, CS; cross-sectional, CTX; C-terminal telopeptide, DHA;
docosahexaenoic acid, DRI; dietary recommended intake, EPA; eicosapentaenoic acid, EPO; evening primrose oil, LA; linoleic acid, NS; non significant, NTX; N-terminal telopeptide, P; prospective, PUFA; polyunsaturated fatty acid, RCT, randomized controlled trial, TNFα; tumor necrosis factor alpha.
Strengths and Limitations of Human Studies

While observational studies and RCTs are clinically relevant, as they directly assess the effect of diet on the human population, there are several limitations associated with these study designs. The use of observational studies in research provides information regarding associations only, and cannot imply causation. The findings from observational studies are also limited by the baseline characteristics of the population studied, the range of intake of a nutrient within the population and the specificity of the dietary assessment tool used, the length of the follow-up period, and the primary outcome measured. Findings from RCTs are similarly affected by factors such as the inclusion criteria and baseline characteristics of the sample population, and the duration of the intervention period and the primary outcome measured. RCTs also provide details of only a snapshot in time in the life of a woman.

Although it is generally understood that habitual dietary patterns of an individual do not change drastically over time (217), studies of the effect of diet in the human population often capture only one specific life stage (i.e., childhood, adulthood, post-menopause). Extending follow-up periods through several life stages in a large sample size would be very costly, but would provide valuable information on the effect of specific lifelong diet patterns to bone health, as bone is a dynamic tissue affected by the chronic exposure to nutrients in the diet, including PUFAs. As an alternative to costly and time-intensive studies in humans, rodent models have been used for the study of bone health in response to dietary interventions. The use of rodent models allows for the assessment of long term feeding trials that can extend over many stages of the life cycle including development, growth, adulthood, aging. Rodent studies also allow for a
researcher to have strict control over factors including diet (type and amount), physical activity and hormone status. Higher resolution imaging and testing of \textit{ex vivo} tissues can also be conducted in a rodent model that cannot be obtained in humans.

\textbf{PUFA and Rodent Studies}

To assess the direct effect of PUFA intake on bone health related outcomes, many studies have been performed using rodent models. These pre-clinical models are beneficial as they provide the unique opportunity to assess the effect of dietary interventions over the life course (i.e., growth and development, attainment of peak bone mass, protection from aging and hormone-related bone loss). Alterations to bone and to bone health is a lifelong process and so early and prolonged exposure to nutrients, such as PUFAs, may provide protection from the hormone-related bone loss experienced later in life, although this hypothesis has never been tested in a rodent model.

\textbf{Feeding Intervention Trials in Growing Rats}

Studies performed on growing rats are summarized in \textbf{Table 2-13} and discussed below.
Table 2-13. Summary of findings from PUFA feeding intervention trials in growing rats

<table>
<thead>
<tr>
<th>Reference</th>
<th>Strain (Sex), Age</th>
<th>Dietary Content (by weight)</th>
<th>Intervention Diets</th>
<th>Duration</th>
<th>Bone-Related Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Claasen, 1995 (156)</td>
<td>SD (M), 4 weeks</td>
<td>8% total fat</td>
<td>Ratio of LA: ALA (3:1) (Control), GLA: EPA+DHA (3:1), GLA: EPA+DHA (1:1), GLA: EPA+DHA (1:3)</td>
<td>6 wk</td>
<td>• GLA: EPA+DHA (3:1) increased calcium balance compared to control (absorption, bone mineral calcium content, excretion)</td>
</tr>
</tbody>
</table>
| Yamada, 1995 (218) | Wistar (M), 6 weeks | 6% total fat               | Soybean oil (6g/kg) (Control), Soybean + EPA (5.4 + 0.6g/kg), Soybean + DHA (5.4 + 0.6g/kg) | 7 wk     | • No effect on femur peak strength  
• EPA-supplementation increased calcium excretion, decreased plasma phosphorous and PTH compared to DHA-supplementation                                                                                   |
| Li, 1999 (219)   | SD (M), 21 days   | 7% total fat               | Soybean oil (Control), Ratio of SFO: menhaden oil (44:56) | 42 d     | • No effect on tibia histomorphometry, or bone calcium, phosphorous, or magnesium content                                                                                                                             |
| Watkins, 2000 (169) | SD (M), 21 days   | 7% total fat               | Ratio of SFO: menhaden oil (90:10), SFO: menhaden oil (80:20), SFO: menhaden oil (50:50), SFO: menhaden oil (30:70) | 42 d     | • Decreasing PUFA ratio associated with increased BALP concentration  
• SFO: menhaden oil (30:70) diet increased bone formation rate compared to (90:10) diet                                                                                                                     |
| Sirois, 2003 (220) | SD (F), 21 days   | 7% total fat               | Soybean oil (7g/kg), Soybean + menhaden oil (1 g/kg soybean + 6 g/kg menhaden oil) | 5 wk     | • No effect on femur BMD, BMC, or biomechanical strength properties  
• Menhaden oil diet decreased LV5 peak                                                                                                                      |
<table>
<thead>
<tr>
<th>Study</th>
<th>Diet Details</th>
<th>Fat Content</th>
<th>Schedule</th>
<th>Load</th>
<th>Findings</th>
</tr>
</thead>
</table>
| Reinwald, 2004 (221) | Long-Evans, 49 days | 7.8% total fat | Modified AIN-93G with coconut and SFO, Modified AIN-93G with 4.8g/kg flaxseed oil + 3.0g/kg DHA | 8 wk | • High n-6 to n-3 ratios in the tibia decreased bone growth, peak load and bending moment  
• No changes to femur mechanical properties |
| Kruger, 2005 (222) | SD (M), 5 weeks | 5% total fat | Corn oil (Control), EPO, Fish oil (4% fish oil, 1% corn oil), Tuna oil (4% tuna oil, 1% corn oil) | 6 wk | • Tuna oil increased calcium balance compared to control (absorption, bone mineral calcium content, excretion)  
• Tuna oil increased lumbar spine and femur BMD compared to control  
• No changes to femur mechanical properties  
• RBC DHA-content associated with lumbar spine and femur BMD and BMC |
| Lau, 2010 (163) | SD (M), 40 days | 20% total fat | SFA diet (18% coconut oil, 2% soybean oil), n-3 diet (20% flaxseed oil), n-6 diet (20% SFO) | 65 d | • No difference in femur mechanical properties at the midpoint or BMC |
| Li, 2010 (165) | Long-Evans (M), PND 2 | 10% total fat | 3.1% of total lipid as ALA (Control), 15% of total lipid as LA, LA diet + 1% of total lipid as DHA, LA diet + 1% of total lipid as DPA, LA diet + 1% of total lipid as DHA + 0.4% as | 15 wk | • All diets reduced distal femur BMC compared to control  
• DPA-supplementation decreased proximal femur BMC compared to control  
• No changes to femur midpoint or tibia bone mass or density |
<table>
<thead>
<tr>
<th>Lukas, 2011 (159)</th>
<th>SD (F), 28 days</th>
<th>12% total fat</th>
<th>Corn oil (73:1) (Control), Flaxseed oil (1:3), Krill oil (1:33), Menhaden oil (1:48), Salmon oil (1:23), Tuna oil (1:12)</th>
<th>8 wk</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Tuna oil increased tibia BMD compared to control</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>• Flaxseed oil and menhaden oil increased femur BV/TV, Tb.N., and connectivity and decreased Tb.Sp. compared to control</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>• Flaxseed increased tibia BV/TV compared to control</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>• Flaxseed oil and menhaden oil increased Tb.N. and connectivity and decreased Tb.Sp. compared to control</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• No changes to femur or tibia cortical microarchitecture or mechanical properties</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Flaxseed oil increased osteocalcin compared to salmon and tuna oil</td>
<td></td>
</tr>
</tbody>
</table>

1 ALA; alpha-linolenic acid, BALP; bone specific alkaline phosphatase, BMC; bone mineral content, BMD; bone mineral density, BV/TV; percent bone volume, d; day, DHA; docosahexaenoic acid, DPA; docosapentaenoic acid, EPA; eicosapentaenoic acid, EPO; evening primrose oil, F; female, GLA; γ-linoleic acid, LA; linoleic acid, M; male, PND; post-natal day, PTH; parathyroid hormone, PUFA, polyunsaturated fatty acid, RBC; red blood cell, SD; Sprague-Dawley, SFA; saturated fatty acid, SFO; safflower oil, wk; week.
Effect of Varying PUFA Source on Bone During Growth

While this thesis focuses on females, many of the studies investigating effects of PUFA source on bone growth have studied male rodents. One of the first studies to determine the effect of different n-6 to n-3 ratios and the impact of essential fatty acids compared to their longer chain derivatives was conducted in 5-week old, male, SD rats (156). Following randomization to a diet consisting of either LA to ALA in a 3:1 ratio or its derivatives γ-linoleic acid to EPA and DHA in a 3:1 ratio (both diets made up of 8% total fat) for 6 weeks, the longer chain PUFA diet caused an increase in intestinal calcium absorption and bone calcium content and a decrease in urinary calcium excretion. These early findings suggest that derivatives of the essential fatty acids may be more effective in modulating calcium balance than their precursors (156). A follow-up study conducted by the same authors also suggested that diets supplemented with γ-linoleic acid and EPA inhibited bone resorption, as measured by urinary collagen cross-link markers, compared to diets supplemented with LA and ALA (160).

To understand the importance of n-3 PUFA in the diet, one study of the offspring of n-3 deficient female Long-Evans rats found that even after n-3 repletion through diet (4.8 + 3.0 g/kg diet of flaxseed oil + DHA), the mechanical properties of the tibia were altered compared to the n-3 adequate controls (221). This suggests that high n-6 to n-3 PUFA ratios fed during the bone modeling process early in life in rapidly growing rats can cause structural changes that ultimately alter bone strength. Another study of young, male rats investigating the effect of the different long chain n-3 PUFA found no effect of feeding diets made up of 6% fat by weight of soybean oil control, soybean oil + EPA, or soybean oil + DHA on femur peak strength (218). EPA-supplementation over 7 weeks
did however increase urinary calcium excretion and decrease plasma phosphorous and parathyroid hormone concentrations compared to rats not receiving EPA. DHA-supplementation did not cause a similar trend in results, suggesting a difference in the effect of the long chain n-3 PUFA on bone related outcomes.

In support of these findings of a benefit of DHA to bone health, supplementation of 5-week, male SD rats for 6 weeks with diets consisting of corn oil, evening primrose oil (high n-6 γ-linoleic acid content), fish oil (high n-3 EPA content), or tuna oil (high n-3 DHA content) suggested diets high in DHA are more effective than EPA in preserving bone mass and increasing calcium bioavailability (222). Tuna oil caused significant increases in calcium absorption, total bone calcium content, lumbar spine and femur BMD compared to corn oil-fed controls. The concentration of DHA in the red blood cell membranes was also significantly correlated with femur and lumbar spine BMD and BMC (222). Together, these results support the effectiveness of DHA over EPA, γ-linoleic acid, and corn oil for bone health related outcomes in young, growing, male rats.

Studies comparing the essential n-3 fatty acid, ALA, and its longer chain derivatives are limited. Weanling female SD rats fed either 7% by weight soybean oil (rich in ALA) or soybean and menhaden oil (1 g soybean oil/kg + 6 g menhaden oil/kg) for 5 weeks did not experience differences in femur BMD, BMC, or biomechanical strength properties, although LV5 peak load was decreased in soybean oil + menhaden oil fed rats (220). Another comprehensive, comparative study determined the effect of feeding n-3 from varying dietary sources to the bone mass, microarchitecture, strength, and markers of formation and resorption in young, growing female rats (159). Twenty-eight day old female SD rats were fed a higher fat diet (12% total fat) composed of corn
oil, flaxseed oil, krill oil, menhaden oil, salmon oil, or tuna oil for 8 weeks. These diets differed in the ALA, EPA, and DHA content and ratio. Only feeding krill oil (highest EPA to DHA ratio of the oil sources) failed to improve any aspect of bone health. Feeding flaxseed (highest ALA content) and menhaden oil (lowest n-6 to n-3 ratio of the oil sources) had positive effects to trabecular microarchitecture of the femur and tibia compared to control, while no diet had an effect on cortical outcomes of the long bones (159). Of the diets tested, flaxseed oil and menhaden oil had the highest ALA content, suggesting that ALA improves the microarchitecture of the long bones in growing female rats. No differences were observed between flaxseed oil and menhaden oil fed groups, possibly due to the large discrepancy in n-6 to n-3 PUFA ratio between these diets (flaxseed oil, 1:3 PUFA ratio versus menhaden oil, 1:48 PUFA ratio). Furthermore, this is the only study to quantify the microarchitecture of the long bones of young rats by μCT in response to PUFA-supplementation. These microarchitecture changes did not correspond to improvements in femur or tibia strength or to calcium or phosphorous balance compared to control. No diet improved both bone mass and bone microarchitecture, highlighting a differential effect of each of the n-3 PUFAs, which may be influenced by the ratio in which they exist in the diet. Studies in this thesis further elucidated the effects of feeding flaxseed oil versus menhaden oil at similar experimentally controlled PUFA ratios on the microarchitecture of the tibia during periods of rapid growth by incorporating these oils in the diets of 28-day old female SD rats and after following their development to young adulthood (1 through 3 months) and for 3 months following OVX-induced bone loss. As discussed previously, exposure to
nutrients over the life course, such as PUFA, may provide protection from the hormone-related bone loss experienced following OVX, although this has never been tested.

To differentiate between the effects of DHA and its n-6 series equivalent, docosapentaenoic acid (DPA, 22:6n-6), Long-Evans rats at postnatal day 2 were either dam-reared by mothers fed a diet consisting of 3.1% of total lipids as ALA or artificially reared with rat milk containing 15% of total lipids as LA (n-3 deficient), LA diet + 1% of total lipids as DHA, LA diet +1% of total lipids DPA, or LA diet + 1% of total lipids as DHA + 0.4% DPA (165). At postnatal day 21, rats were weaned onto pelleted diets of the same treatment type and following 15 weeks of feeding, a trend towards lower BMD and BMC of the femur, tibia, and LV from rats fed DPA was observed, although these results were not statistically significant. Of importance to note in this study, no differences were observed in outcomes measured by DXA between rats randomized to a diet of ALA (3.1% of total lipids) or a diet of DHA (1% of total lipids), although those receiving ALA had three-fold greater n-3 PUFA by weight in the diet (165). These results suggest that the tibia, femur, and LV are able to adapt to the PUFA source incorporated in the diet at very early life and regardless of diet, the bones are able to develop similarly. The effect of PUFA source on bone structure by µCT was not examined in this study.

**Effect of Varying PUFA Ratio on Bone During Growth**

*While feeding intervention trials in animals allow for the strict manipulation of specific dietary components to understand the impact of a single PUFA source, what may be more reflective of the human diet is the study of the n-6 to n-3 PUFA ratio on bone health.* To elucidate the effect of varying ratio of n-6 to n-3 PUFA on the bone health,
two studies have used young, growing, 21-day old male SD rats randomized to receive diets of varying n-6 to n-3 ratios for 42-days (169, 219). Fat from these diets made up 70 g/kg of total diet and the ratio of PUFA were altered with the addition of LA and EPA/DHA from safflower oil and menhaden oil, respectively. In the first of these studies, soybean oil control (n-6 to n-3 PUFA ratio of approximately 6.5:1) caused an increase in *ex vivo* PGE$_2$ production from tibia and femur bone culture compared to safflower oil and menhaden in a 44:56 ratio by weight but no changes in the histomorphometry of the tibia were observed (219). In a follow-up study from the same authors, rats were randomized to receive 1 of 4 diets made up of safflower oil and menhaden oil ratios by weight in the following proportions: (90:10), (80:20), (50:50), (30:70) (169). These proportions resulted in dietary n-6 to n-3 PUFA ratios of 23.8, 9.8, 2.6, and 1.2 to 1, respectively. The commonly used control diet, AIN-93G, provides 7% fat by weight from soybean oil, and provides a PUFA ratio of 6.5:1. Of importance to note, the total PUFA of each diet was decreased with the decreasing ratio, which may have impacted the results of this study. With a decreasing n-6 to n-3 ratio in the diet, an increase in the bone formation marker bone alkaline phosphatase was observed. 

Histomorphometric analysis of the proximal tibia resulted in significant increase in bone formation rate between diets of the highest and lowest PUFA ratio. *Ex vivo* PGE$_2$ production from the rat tibia was negatively correlated with bone formation rates, which supports a positive effect of low n-6 to n-3 PUFA ratios in the diet of young, growing male rats for improved bone outcomes by decreasing PGE$_2$ signaling (169, 219). Changes to bone structure with altered dietary PUFA ratio was not measured in either of these studies and remains unknown.
Effect of High-Fat PUFA Diet on Bone During Growth

While most studies of young, growing rats involve the impact of low to moderate fat diets, one study has investigated the impact of high-fat diets (20% fat by weight) with fats from coconut oil (saturated fat), flaxseed oil (n-3 PUFA in the form of ALA), or safflower oil (n-6 PUFA in the form of LA) compared to chow-fed rats (5.2% fat by weight) on bone related outcomes in 40-day old male SD rats (163). Following 65 days of feeding, the fatty acid composition of the femur reflected the fatty acid composition of the diet but these changes did not alter femur BMD or biomechanical strength properties of the femur midpoint between rats fed the high-fat diets (163). High-fat diet feeding caused significant increases in the unadjusted biomechanical strength properties: yield load, resilience, stiffness, peak load, toughness and the BMD and BMC of the femur compared to normal chow-fed rats. After adjustment for body weight, all differences were lost except, rats fed n-3 PUFA or n-6 PUFA rich diets experienced greater peak load at the femur midpoint, suggesting some benefit to these high-fat PUFA rich diets in this cortical bone site.

Summary of Findings in Growing Rats

Most studies of feeding intervention trials in young, growing rats have shown some benefit to bone health related outcomes during this period of rapid growth and bone modeling, although only one study has been conducted in female rats. Growing female rats reach sexual maturity at 6 weeks of age (223), and so there may be potential sex differences between males and females and the effect of feeding interventions on bone-related outcomes by this age. Several studies have suggested a benefit with consumption of a lower n-6 to n-3 PUFA ratio (159, 169, 221), but the source of n-3 PUFA seems to
have an effect. Generally, a benefit to bone has been observed with DHA-supplementation (159, 165, 222) and a detriment with EPA-supplementation (159, 218) suggesting that the long chain n-3 PUFA have different effects when fed to growing rats.

Only two studies have directly compared the effect of feeding ALA from flaxseed to other fat sources in young rats (159, 163). Feeding diets with 20% and 12% fat from flaxseed does not affect bone strength compared to coconut oil or safflower oil or compared to marine sources of long chain n-3 PUFAs, respectively (159, 163), but the latter diet supplemented for 8 weeks did result in changes to bone microarchitecture (159). Only one study to date has investigated the effect of ALA-supplementation from flaxseed compared to similar amounts of the long chain n-3 PUFA, EPA and DHA, from fish oil sources. Although this study furthers our understanding of PUFA and bone health, the quantities and ratios implemented in this feeding trial are supra-physiological and do not represent typical human consumption patterns (159). The average Western diet provides n-6 to n-3 PUFA ratios of roughly 15 through 17:1 (155), but the studies reviewed above provide a range of PUFA ratios from 73:1 to as low as 1:38 (159).

While several studies have investigated the impact of feeding intervention trials on the growth and development of bone tissue, the effect of similar nutritional interventions to provide potential protection against OVX-induced bone loss have also been studied. To date, no studies have followed the same rat longitudinally to examine the effect of lifelong exposure to PUFA interventions through growth, young adulthood, and following OVX. Therefore, this was a primary objective of Study 4 of this thesis.

Feeding Intervention Trials in OVX Rats
Several animal studies have been conducted using the OVX rat as a pre-clinical model of postmenopausal osteoporosis to determine the effect of dietary PUFA intake on bone health related outcomes. Studies performed on OVX rats are summarized in Table 2-14 and discussed below.
Table 2-14. Summary of findings from PUFA feeding intervention trials in OVX rats

<table>
<thead>
<tr>
<th>Reference</th>
<th>Age at OVX (Strain)</th>
<th>Dietary Content (by weight)</th>
<th>Intervention Diets</th>
<th>Duration</th>
<th>Bone-Related Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sakaguchi, 1994 (224)</td>
<td>17 wk (SD)</td>
<td>3.4% from fat</td>
<td>Corn oil (control), Corn oil low calcium, 3.1% Corn oil + 0.3% EPA, 3.1% Corn oil + 0.3% EPA low calcium</td>
<td>35 days</td>
<td>• EPA-supplementation increased femur midpoint strength when calcium is deficient</td>
</tr>
<tr>
<td>Kruger, 1997 (225)</td>
<td>11 wk (SD)</td>
<td>8% from fat</td>
<td>LA: ALA (3:1) (control), GLA: EPA+DHA (9:1), GLA: EPA+DHA (3:1), GLA: EPA+DHA (1:3), GLA: EPA+DHA (1:9)</td>
<td>6 weeks (initiated 1 week post-OVX)</td>
<td>• RBC DGLA concentrations negative correlated with urinary deoxypyridinoline • RBC EPA, DGLA, and DHA concentrations positively correlated with femur calcium content</td>
</tr>
<tr>
<td>Schlemmer, 1999 (226)</td>
<td>11 wk (SD)</td>
<td>1% from fat</td>
<td>1% Sunflower oil (control), 1% Sunflower oil + 1g/kg GLA+EPA</td>
<td>3 weeks</td>
<td>• No effect on femur length or calcium content of the femur • No effect on deoxypyridinoline or osteocalcin concentrations</td>
</tr>
<tr>
<td>Watkins, 2005 (170)</td>
<td>2 mo (SD)</td>
<td>11% from fat</td>
<td>100% from SFO (344:1), 72.6% from SFO + 27.4% from menhaden oil (5.6:1)</td>
<td>12 weeks (initiated 1 week post-OVX)</td>
<td>• Lower PUFA ratio increased tibia BMC in vivo and ex vivo • Lower PUFA ratio increased femur and tibia length • No effect on serum biomarkers or histomorphometry of the tibia</td>
</tr>
<tr>
<td>Poulsen, 2006 (227)</td>
<td>8 mo (SD)</td>
<td>4% from fat</td>
<td>Corn oil (control), 3.8% Corn oil + 0.2% EPA (0.1 mg/kg body weight), 2.2% Corn oil + 1.8% EPA (1.0 mg/kg body weight)</td>
<td>10 weeks</td>
<td>• High dose EPA-supplementation reduced femur BMC and BMD compared to control • No change in LV BMC or BMD • High dose EPA-supplementation increased CTX-1 concentrations compared to Sham</td>
</tr>
<tr>
<td>Study</td>
<td>Duration</td>
<td>Fat Source</td>
<td>Dietary Intervention</td>
<td>Intervention Details</td>
<td>Study Duration</td>
</tr>
<tr>
<td>-------------------------------</td>
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</tbody>
</table>
| Watkins, 2006 (141)           | 3 mo (SD)| 11% from fat | 76% from SFO + 24% from DHA (5:1), 87% from SFO + 13% from DHA (10:1), 87% from high-oleate SFO + 13% from DHA (5:1), 93% from high-oleate SFO + 7% from DHA (10:1) | 12 weeks | • Lower PUFA ratio increased femur BMC  
• Higher PUFA content in the diet increased serum pyridinoline and deoxypyridinoline and decreased osteocalcin concentrations compared to low PUFA diets |
| Poulsen, 2007 (166)           | 7 mo (SD)| 4% from fat | Corn oil (control), 3.3% Corn oil + 0.7% GLA, 3.3% Corn oil + 0.7% EPA, 3.3% Corn oil + 0.7% DHA, 3.3% Corn oil + 0.2% GLA + 0.1% EPA + 0.4% DHA | 16 weeks | • No change in LV or femur BMC or BMD compared to control  
• DHA-supplementation decreased LV BMC percent change from baseline and increased tibia endosteal circumference compared to control  
• GLA-supplementation increased femur BMC percent change from baseline and tibia trabecular bone area and decreased break stress compared to control |
| Poulsen, 2008 (228)           | 7 mo (SD)| 4% from fat | Corn oil (control), 3.3% Corn oil + 0.7% DHA | 18 weeks | • DHA-supplementation increased femur BMC and BMD compared to control (DXA)  
• No effect on biomechanical properties of the femur  
• No effect on LV BMC and BMD  
• DHA-supplementation increased trabecular BMC of the tibia (high-resolution pQCT) |
<p>| Boulbaroud, 2008 (229)        | 3 mo (Wistar) | 7 or 10% from fat | Corn oil (control), Sesame oil, Flaxseed oil | 4 weeks | • 10% sesame oil and flaxseed oil supplementation reduced serum BALP and TRAP activity |
| Matsushita, 2008              | 12 mo    | 4% from fat | Soybean oil (control), | 10 weeks | • Menhaden oil increased whole femur BMC |</p>
<table>
<thead>
<tr>
<th>Year</th>
<th>Duration</th>
<th>Treatment</th>
<th>Study Duration</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>2008 (230)</td>
<td>retired breeder (SD)</td>
<td>Menhaden oil</td>
<td></td>
<td>and BMD (distal femur) and percent mineralizing surface and mineral apposition rate on endocortical surfaces of the femur in OVX rats only</td>
</tr>
<tr>
<td>Sacco, 2009 (139)</td>
<td>3 mo (SD)</td>
<td>4% Soybean oil (4%) (control), 4% Soybean oil + 10% Flaxseed</td>
<td>12 weeks</td>
<td>• No effect on serum CTX concentrations</td>
</tr>
<tr>
<td>Sacco, 2014 (231)</td>
<td>3 mo (SD)</td>
<td>4% Soybean oil (4%) (control), 4% Soybean oil + 10% Flaxseed</td>
<td>2 weeks, 12 weeks</td>
<td>• Flaxseed-supplementation increased OPG concentrations (2 weeks)</td>
</tr>
</tbody>
</table>

Flaxseed increased osterix-positive cells compared to control (12 weeks)  
• No change in LV trabecular microarchitecture (μCT) (12 weeks)

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1 ALA; alpha-linolenic acid, BALP; bone specific alkaline phosphatase, BMC; bone mineral content, BMD; bone mineral density, CTX; C-terminal telopeptide, DGLA; dihomo-γ-linoleic acid, DHA; docosahexaenoic acid, DXA; dual energy X-ray absorptiometry, EPA; eicosapentaenoic acid, GLA; γ-linoleic acid, LA; linoleic acid, LV; lumbar vertebrae, mo; month, OPG; osteoprotegerin, OVX; ovariectomy, pQCT; peripheral quantitative computed tomography, PUFA; polyunsaturated fatty acid, RBC; red blood cell, SD; Sprague-Dawley, SFO; safflower oil, TRAP; tartrate-resistant acid phosphatase, μCT; micro-computed tomography, wk; week.
Effect of Varying PUFA Source on Bone in OVX Rats

To examine the specific effects of PUFA source on bone health, one short-term study of 11-week OVX SD rats supplemented either 1% sunflower oil (high LA content) or 1% sunflower oil + 1g/kg γ-linoleic acid +EPA diester (226). In this study, γ-linoleic acid +EPA supplementation did not alter serum markers of bone turnover or urinary markers of collagen breakdown and had no effect on femur calcium content or femur length. The lack of effects may be due to the short duration of feeding following OVX, as rats were only supplemented with their respective diet for 3-weeks post-OVX. The lack of effects may also be due to the low fat content in the diets.

One of the first studies conducted to compare the effect of EPA with and without adequate amounts of calcium in the diet to a corn oil diet control suggests a benefit to EPA-supplementation (224). EPA levels as low as 0.3% of total energy intake (with 3.1% total energy from corn oil) were sufficient to rescue femur midpoint peak strength and femur wet weight in female SD rats over 35 days when calcium intake was deficient (0.01% calcium) but did not provide added benefit when dietary calcium was adequate (1.26% calcium) (224). In contrast, a study evaluating the effect of 10-week supplementation with a low (3.8% corn oil + 0.2% EPA) or high (2.2% corn oil + 1.8% EPA) EPA diet on 8-month OVX SD rats reported a detrimental effect of high dose EPA (227). To limit any baseline variations between groups, the authors of this study provided all groups with 0.5% calcium in the diet, and 4-weeks prior to OVX or Sham-surgery, all groups were fed the 4% corn oil control diet. With a higher EPA-supplemented diet, a decrease in femur BMD and BMC was observed compared to OVX control-fed rats (4% fat from corn oil) at study endpoint. High-dose EPA groups
experienced the greatest reduction in percent BMD and BMC of the femur and LV,
suggesting a diet of 2.2% corn oil with 1.8% EPA exacerbates the deleterious effects following OVX compared to corn oil control (227). A follow-up study of 7-month OVX SD rats fed either control diet with 4% corn oil or 3.3% corn oil + 0.7% from either γ-linoleic acid, EPA, DHA, or a combination for 16-weeks found γ-linoleic acid-supplementation reduced break stress (N/mm$^2$) of the femur but increased trabecular area of the tibia compared to control-fed OVX rats (166). γ-linoleic acid-supplementation in the diet exacerbated the percent change of femur BMD from baseline to endpoint, while DHA-supplementation reduced the percent change in LV BMC compared to control rats. These diets differed not only in their PUFA source, but also the PUFA ratio. γ-linoleic acid diets provided rats with a 31:1 ratio of n-6 to n-3 PUFA, while DHA diets provided a 4.5:1 PUFA ratio. DHA and EPA-supplementation each increased endosteal circumference of the tibia, suggesting a benefit to cortical bone with long chain n-3 PUFA feeding. Further investigation of the effect of DHA-supplementation in 7-month OVX SD rats found increased femur BMC and BMD as measured by DXA and increased tibia trabecular BMC as measured by high-resolution pQCT compared to control-fed OVX rats (4% corn oil compared to 3.3% corn oil + 0.7% DHA for 18 weeks) (228). These changes did not however result in changes to mechanical properties of the femur. Together, these findings highlight a benefit of DHA incorporation in the diet to the trabecular bone following OVX.

Much work has focused on the effect of the long chain n-3 PUFA on bone health, but only three studies in the OVX rat have investigated the effect of the essential n-3 PUFA, ALA (139, 229, 231). Following a 4-week supplementation period in 3-month
Wistar rats fed 10% fat by weight from corn oil control, flaxseed oil, or sesame oil, both intervention diets reduced serum bone alkaline phosphatase levels and tartrate-resistant acid phosphatase activity, suggesting an overall effect of PUFA on bone cell activity (229). Interestingly, similar results were not observed when these fats were incorporated as 7% by weight (229). Both as part of larger studies, 3-month SD rats provided with diets supplemented with 10% flaxseed were compared to their AIN-93M fed controls (4% fat from soybean oil). Following 2-weeks of supplementation, a sufficient time point for monitoring changes to bone metabolism, rats receiving flaxseed experienced higher serum OPG concentrations compared to control, but no changes in other biomarkers of bone turnover were observed (231). Twelve-week feeding of 10% flaxseed diets did not alter BMD of biomechanical strength properties of the lumbar spine, femur, or tibia (139), or µCT-related trabecular outcomes of LV (231). Flaxseed supplementation did however increase the number of osterix-positive cells compared to control, suggesting a higher osteoblast number (231). These results were part of a larger study, which found that flaxseed supplementation acts synergistically with low dose estrogen to provide metabolic and structural benefits to bone following OVX-induced bone loss.

**Effect of Varying PUFA Ratio on Bone in OVX Rats**

To assess the effect of dietary PUFA ratio, a 12-week study of 3-month OVX SD rats was undertaken (141). In this study, OVX rats were randomized to 1 of 5 diets with 25% of energy derived from fat (11% total fat by weight). The diets had either a high PUFA content (68% of total fat from PUFA) or low PUFA content (37% of total fat from PUFA) and a PUFA ratio of either 10:1 or 5:1, with almost all of the n-3 PUFA derived
from DHA. Feeding a 5:1 PUFA ratio resulted in greater femur BMC compared to rats fed a 10:1 ratio when measured both in vivo and ex vivo and the ratio of tissue LA to total n-3 was negatively correlated with femur BMC, suggesting that lowering the PUFA ratio of the diet may play a significant role in minimizing bone mineral loss in the femur following OVX when n-3 PUFA is provided as DHA (141). Total PUFA intake did not have an effect on femur bone mineral, but high PUFA intake caused a significant decrease in osteocalcin and an increase in serum pyridinoline and deoxypyridinoline compared to low total PUFA intake. This suggests that diets lower in total PUFA content promote bone formation and reduce markers of resorption (141). With the design of this study, the authors were able to investigate the effect of both total PUFA and PUFA ratio in the OVX model and found beneficial effects to feeding lower total PUFA and to feeding lower PUFA ratios. Although total fat was matched, total saturated fatty acid (SFA) and total monounsaturated fatty acids (MUFA) varied across all diets.

Other studies investigating the role of PUFA ratio and bone health have yielded similar results supporting the benefit of a low PUFA ratio and the preservation of bone health (170, 225), although very few studies have investigated the effect on microarchitecture by μCT. In one study of 12-week OVX SD rats randomized to receive a control diet consisting of LA: ALA in a 3:1 ratio, or γ-linoleic acid: EPA+DHA in a 9:1, 3:1, 1:3, or 1:9 ratio with all fat contributing to 8% of the diet by weight, red blood cell concentrations of dihomo-γ-linoleic acid (the downstream derivative of γ-linoleic acid), EPA, and DHA were significantly correlated with calcium content of the femur (225). Only dihomo-γ-linoleic acid concentrations were negatively correlated with urinary deoxypyridinoline concentrations, however. Comparison of 2-month OVX SD
rats supplemented for 12 weeks with a PUFA ratio of 344:1 (with 100% of fat from safflower oil) or 5.6:1 ratio (with 72% fat from safflower oil and 27% fat from menhaden oil) resulted in benefits to the femur and tibia with lower PUFA ratios (170). While these results support a benefit to bone health following OVX with feeding lower dietary PUFA ratios, these studies utilize PUFA ratios that are not representative of a typical human dietary pattern. For this thesis, the dietary ratio of PUFA was altered to more closely mimic human PUFA intakes (10:1 versus 5:1) and fed to female SD rats to further elucidate the effect of varying PUFA ratio on the trabecular and cortical microarchitecture of bone (Study 4).

**PUFA Effects in Sham and OVX Rats Differ**

In most studies of the OVX rat model, the effect of PUFA intervention diets are only assessed in OVX animals and all results are compared to one group of Sham-operated and OVX rats receiving control diet. This strategy is used to minimize groups and overall animal numbers, but there is evidence that bone from Sham-operated/control rats and from OVX rats does not respond to feeding trials of PUFA similarly (230, 232). Study 4 of this thesis determined the differential effects of PUFA exposure over the life course in Sham and OVX rats to bone microarchitecture outcomes of the proximal tibia.

To assess the effect of soybean oil compared to menhaden oil, 12-month retired breeder SD rats were fed diets with 4% of energy derived from soybean or from menhaden oil for 10-weeks following Sham-control or OVX (230). Menhaden oil supplementation caused increases in femur BMC and BMD (distal femur only) measured by DXA compared to their soybean-fed OVX controls. Menhaden oil supplementation in OVX rats also caused increased rates of mineral apposition and percent mineralized
surface on the endocortical surfaces of the femur midpoint compared to OVX controls. No differences in serum CTX concentrations, a marker of bone resorption was observed (230), suggesting menhaden oil causes an increased rate of bone formation but not resorption in OVX rats. These results were not observed when menhaden oil was supplemented in Sham-operated rats compared to soybean oil control, suggesting a beneficial role for long chain n-3 PUFAs only in circumstances of estrogen deficiency induced bone loss.

Similarly, supplementation with 15% soybean oil or 10% sesame oil for 8-weeks in OVX SD rats caused changes to serum calcium, phosphorus, magnesium, estrogen, parathyroid hormone, osteocalcin, and bone alkaline phosphatase but no effect of diet was observed in normal control rats fed similar diets (232). A similar pattern of an effect of soybean and sesame oil supplementation in OVX rats only was found for femur BMD, calcium, phosphorous, and magnesium concentrations, and bone alkaline phosphatase activity. Although findings from this study are interesting, the differences between normal and OVX rats cannot be directly compared as two species of rats were used (normal control Wistar rats compared to OVX SD rats) and the quantity of fat in the diet was not controlled across experimental groups (232). These studies did not quantify the effects of dietary PUFA intake on bone microarchitecture outcomes, and so whether a differential effect of PUFA intake on Sham/control and OVX rats exists is unknown.

**Summary of Findings in OVX Rats**

Similar to findings in growing rats, feeding intervention trials of the OVX rat have found a benefit with DHA-supplementation (141, 166) and a detriment with EPA-supplementation (227) to bone health. Limited studies of the essential n-3 fatty acid,
ALA have been conducted in OVX rats (139, 229, 231). Moreover, no study has directly compared the effect of sources rich in ALA (i.e., flaxseed oil) with oils rich in its long chain derivatives, EPA and DHA, in the OVX rat. Lowering the n-6 to n-3 PUFA ratio in the diet provides benefits to bone health related outcomes in OVX rats (141, 170, 225), although some studies provide PUFA ratios in the diet that are supra-physiological and do not represent dietary patterns that can be sustained in humans (170, 225).

To limit any differences at baseline, which may confound some of the changes induced by diet, many studies have incorporated a period of feeding a control diet prior to randomization (139, 156, 166, 226, 227, 231). This period of pre-feeding ranges from 1 to 4 weeks in the literature and provides an added level of control in these studies of OVX rats. While this strategy limits any baseline variations between groups, this thesis is the first to investigate the potential protective effect of a pre-diet of varying PUFA source and ratio on bone health following OVX.

**Summary of Findings of Feeding Intervention Trials in Rats**

Many studies have been conducted in rats to assess the effect of feeding various quantities and sources of PUFA, and generally, a benefit to bone health outcomes has been observed when compared to control diets, although very few studies have assessed changes to bone microarchitecture through µCT. These studies can be categorized based on the life stage of the rat when the intervention is applied: during growth and bone modeling or following OVX and bone remodeling. But, no study to date has combined these life stages and followed the response of a rat to a PUFA-feeding intervention throughout growth and in response to Sham or OVX surgery. As benefits to bone health with PUFA-incorporation in the diet have been observed in growing rats, it can be
hypothesized that lifelong PUFA-supplementation may provide a protection to the age- and hormone-associated decline in bone health in older, OVX rats.
Chapter 3: Objectives and Hypotheses
Objectives

The overall objective of this thesis is to characterize the µCT scanning method used to evaluate the changes to bone structure following OVX and exposure to a PUFA intervention over the life course in a rat model of postmenopausal osteoporosis.

The specific objectives of this thesis are four-fold and uses a female rat model:

1. To characterize the longitudinal changes in the proximal tibia microarchitecture over a 3-month period, following Sham or OVX-surgery at 3-months of age (Study 1).

2. To determine if monthly repeated radiation exposure of the hind limb in Sham and OVX rats over a 3-month period alters the trabecular or cortical microarchitecture of the proximal tibia (Study 2).

3. In rat proximal tibia, i) to determine the differences in bone structure from *in vivo* compared to *ex vivo* scans; ii) to determine how voxel size (9µm versus 18µm) of *ex vivo* scans modulated the quantification of bone structure; iii) to determine if the well characterized differences between Sham and OVX groups can be similarly detected with each scan protocol (Study 3).

4. To determine how the dietary source of n-3 PUFA (ALA from flaxseed oil versus EPA and DHA from menhaden oil) and dietary ratio of n-6 to n-3 PUFA (10:1 versus 5:1) modulate outcomes of bone health when fed from 1 month of age through to 6 months of age, following Sham or OVX surgery at 3 months of age (Study 4).
Hypotheses

1. OVX rats but not Sham rats, will experience characteristic changes to trabecular and cortical microarchitecture of the proximal tibia including decreased BV/TV, Tb.N., Tb.Th., Ct.Th., Ec.Pm., Ct.Ar./Tt.Ar., and increased Tb.Sp., Ma.Ar., Ps.Pm. over a 3-month period (Study 1).

2. Monthly repeated radiation exposure to Sham and OVX rats over a 3-month period will not alter the trabecular or cortical microarchitecture of the proximal tibia (Study 2).

3. i) Scanning of the proximal tibia in vivo and ex vivo will not alter the quantification of trabecular or cortical microarchitecture in Sham or OVX rats
   ii) Scanning of the proximal tibia at a different voxel size (18µm versus 9µm) will alter the absolute quantification of trabecular and cortical microarchitecture parameters.
   iii) The expected difference between Sham and OVX groups will be detected similarly, regardless of scan protocol (Study 3).

4. i) Feeding menhaden oil from 1 through 3 months of age will cause changes, generally considered to be beneficial, to microarchitecture outcomes of the proximal tibia compared to rats fed flaxseed oil.
   ii) Feeding menhaden oil from 1 through 6 months of age will cause changes, generally considered to be beneficial, to microarchitecture outcomes of the proximal tibia following Sham or OVX surgery at 3 months of age compared to rats fed flaxseed oil.
iii) Feeding a PUFA ratio of 5:1 from 1 through 3 months of age will cause changes, generally considered to be beneficial, to microarchitecture outcomes of the proximal tibia compared to rats fed a PUFA ratio of 10:1.

iv) Feeding a PUFA ratio of 5:1 from 1 through 6 months of age will cause changes, generally considered to be beneficial, to microarchitecture outcomes of the proximal tibia following Sham or OVX surgery at 3 months of age compared to rats fed a PUFA ratio of 10:1 (*Study 4*).
Chapter 4: Experimental Approach
Experimental Approach

For this thesis, female rats were fed a diet of varying PUFA ratio and n-3 PUFA source from early life and following randomization to Sham- or OVX-surgery. By measuring the changes to the trabecular and cortical microarchitecture and BMD of the rat at various stages of life and bone development the potential protective effect of PUFA incorporation in the diet, at levels representative of human dietary patterns was assessed.

As outlined in Chapter 2, several knowledge gaps exist in the literature surrounding the changes to the trabecular and the cortical microarchitecture of the long bones, as measured by μCT, in response to endogenous estrogen deficiency and to varying PUFA quantity and source within the diet at levels attainable through common dietary practice (i.e., not through supplementation). This thesis fills these knowledge gaps through characterization and experimentation with the female SD rat either Sham-operated or having undergone OVX at 3 months, coinciding with periods of bone modeling and remodeling (Figure 4-1).
Study 1
- Sham versus OVX at 3 mo
- Longitudinal in vivo μCT assessment at 3, 4, 5, 6 months
- Trabecular and cortical microarchitecture of the proximal tibia

Study 2
- Sham versus OVX at 3 mo
- Repeated in vivo μCT radiation exposure at 3, 4, 5, 6 months (right) versus single radiation exposure at 6 months (left)
- Trabecular and cortical microarchitecture of the proximal tibia

Study 3
- Sham versus OVX at 3 mo
- in vivo versus ex vivo μCT assessment at 18μm resolution
- 9μm versus 18μm resolution (ex vivo)
- Trabecular and cortical microarchitecture of the proximal tibia

Use of μCT for the characterization of OVX rat model of PM osteoporosis

Use of μCT for the evaluation of lifelong PUFA-supplementation

Study 4
- Varying PUFA Source: ALA from flaxseed versus EPA+DHA from menhaden oil
- Varying PUFA Ratio: 10:1 versus 5:1
- PUFA-supplementation from 1 mo (growth, modeling) to 6 mo (adulthood, remodeling)
- Sham versus OVX at 3 mo
- Trabecular and cortical microarchitecture of the proximal tibia at 3 and 6 mo, and LV at 6 mo
- OPG and RANKL serum concentrations
- In vivo body composition by μCT at 6 mo

**Figure 4-1. Summary of the experimental approach used to characterize the changes to bone microarchitecture in the rat model of postmenopausal osteoporosis by μCT and the investigation of lifelong PUFA-supplementation and bone health**

Study 1, 2, and 3 characterized the OVX rat model of postmenopausal osteoporosis through μCT. Study 1 characterized the longitudinal changes to trabecular and cortical bone microarchitecture following Sham or OVX-surgery at 3 months of age in the rat. Study 2 and 3 evaluated the effects of scanning protocols (i.e., repeated irradiation, in vivo, ex vivo, pixel size) on the bone microarchitecture. Study 4 investigated the effect of lifelong exposure to varying n-6 to n-3 PUFA ratio and n-3 PUFA source to bone microarchitecture, serum markers of bone formation and bone resorption, and to body composition.
Study 1: Time-course of the changes to bone microarchitecture in the 3-month OVX rat model of postmenopausal osteoporosis

To characterize the longitudinal changes to bone microarchitecture during periods of modeling and remodeling, Study 1 assessed bone microarchitecture at monthly intervals over a period of 3 months in the female SD rat having undergone OVX or Sham-surgery at 3-months of age. Although much work has been done in older SD rats, this study further characterizes the changes to the trabecular microarchitecture of younger rats, often used in nutritional and/or drug intervention studies for practical reasons discussed in Chapter 2. Results from Study 1 added to the limited knowledge of longitudinal changes to in vivo cortical microarchitecture of the tibia.

Study 2: Characterization of the effect of repeated radiation exposure by in vivo µCT scanning in rat tibia

With the development of the µCT and the ability to repeatedly image an animal in vivo, the potential effect of repeated X-ray radiation exposure to the very skeletal site under investigation must be elucidated. Study 2 determined the effect of monthly repeated radiation exposure by µCT using the SkyScan 1176 scanner in 3-month old Sham and OVX SD rats to their contralateral hind limb, irradiated at study endpoint only. Findings from this study provided insight into whether changes observed over time are due to the intervention and not due to repeated radiation-induced effects.

Study 3: Comparison of ex vivo and in vivo µCT of rat tibia at different scanning settings

Multiple considerations must be made during the experimental design of in vivo studies of animal models including primary outcome, time, cost, and animal welfare. To reduce the cost and the time-sensitive nature of endpoint in vivo µCT scanning, Study 3 investigated the difference between in vivo and ex vivo scanning of the tibia. Secondary
to this, the effect of \textit{ex vivo} scanning of the tibia with voxel sizes of 18\textmu m and 9\textmu m were compared to determine the effect of voxel size on trabecular and cortical outcomes of the proximal tibia in Sham and OVX rats.

\textbf{Study 4: Lifelong intake of flaxseed or menhaden oil to provide varying n-6 to n-3 PUFA ratios modulate bone microarchitecture during growth, but not after OVX}

Following characterization of the OVX model and the \textit{\mu CT} method for quantification of trabecular and cortical microarchitecture of the proximal tibia, the effect of PUFA-supplementation in the diet was assessed. Young (28-day old) female SD rats were randomized to receive diets with varying PUFA ratio (10:1 versus 5:1) and with varying n-3 PUFA source (ALA from flaxseed oil versus EPA and DHA from menhaden oil). Prior to randomization to Sham or OVX-surgery at 3 months of age, rats underwent an \textit{in vivo} \textit{\mu CT} scan of the right proximal tibia. To assess the effect of varying PUFA diets in response to Sham or OVX surgery, rats were maintained on their respective diet and underwent an \textit{in vivo} \textit{\mu CT} scan of the proximal tibia and of the body cavity surrounding LV3 at 6 months of age, coinciding with study endpoint. \textit{Ex vivo} \textit{\mu CT} scanning of LV4 was done to assess an effect of diet at a second, non load-bearing skeletal site. Trabecular and cortical microarchitecture were quantified from the \textit{in vivo} and \textit{ex vivo} \textit{\mu CT} scans and \textit{in vivo} body composition of the abdominal region surrounding LV3 was quantified. At study endpoint, a serum marker of bone formation and bone resorption was quantified by multiplex enzyme assay.
Materials and Methods

All studies were approved by the Animal Care Committee of Brock University and conducted in accordance with the guidelines established by the Canadian Council on Animal Care (Study 1 and 2, AUP 13-11-01; Study 3 and 4, AUP 14-09-02).

Animals, Food Intake, Body Weight, Diet

For Study 1 and 2, female virgin SD rats underwent Sham-control or OVX surgery at 12-weeks of age (n=12/group) and were obtained and entered into the studies following a 7-day recovery and acclimatization period from Charles River Canada (QC, Canada). Rats were doubly housed with cage-mates of similar surgery type in a controlled environment (12-h light/dark cycle; 20ºC). Rats were given ad libitum access to a control diet (AIN-93M, Harlan Teklad, Mississauga, Ont) and water. Food intake was measured twice per week and body weight was measured weekly for the duration of the study. Rats were sacrificed at 6 months of age. Success of OVX was confirmed at necropsy by the visual determination of atrophy of the uterine horns.

For Study 3 and 4, 21-day old female virgin SD rats (n=120) were obtained from Charles River Canada (QC, Canada) and given ad libitum access to a control diet (AIN-93G, Harlan Teklad, Mississauga, Ont) and water. Rats were doubly housed in a controlled environment (12-h light/dark cycle; 20ºC). Following a 7-day acclimatization period, rats were randomized to 1 of 4 dietary treatments modified from AIN-93G to contain 18% kcal from protein, 57% kcal from carbohydrate, and 25% kcal from fat. Diets were modified to contain an n-6 to n-3 ratio of 10:1 or 5:1 with LA provided primarily from safflower oil as the n-6 source and ALA provided from flaxseed oil or EPA and DHA provided from menhaden oil as the n-3 source. Total SFA, MUFA, and
PUFA were matched in all diets (Table 4-1). Rats were given *ad libitum* access to food and water from 1 through 6 months of age. At 3-months of age, rats were randomized to receive Sham-control or OVX surgery (n=12-13/group/diet) or to be sacrificed for baseline tissue collection (n=4-6/diet). Body weight was measured weekly and food intake was measured three-times weekly. To avoid oxidation, all diet was stored in vacuum-sealed bags at -20°C until use and were changed twice per week. Rats were sacrificed at 6 months of age (n=96). Success of OVX was confirmed at necropsy by comparison of uterine weight between Sham and OVX groups.

Sham (n=8) and OVX (n=8) rats receiving a diet modified to contain an n-6 to n-3 ratio of 10:1 with safflower oil as the primary n-6 source and EPA and DHA from menhaden oil as the primary n-3 source were used for Study 3.
Table 4-1. Dietary composition of four experimental diets used in Study 4 $^{1,2}$

<table>
<thead>
<tr>
<th>Component (g/kg)</th>
<th>Flaxseed oil</th>
<th></th>
<th>Menhaden oil</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10:1</td>
<td>5:1</td>
<td>10:1</td>
<td>5:1</td>
</tr>
<tr>
<td>Casein + L-Cysteine</td>
<td>203</td>
<td>203</td>
<td>203</td>
<td>203</td>
</tr>
<tr>
<td>Corn Starch</td>
<td>357</td>
<td>357</td>
<td>357</td>
<td>357</td>
</tr>
<tr>
<td>Maltodextrin</td>
<td>132</td>
<td>132</td>
<td>132</td>
<td>132</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>Mineral Mix</td>
<td>35</td>
<td>35</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin Mix</td>
<td>10</td>
<td>10</td>
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<td>10</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
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<td>2.5</td>
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</tr>
<tr>
<td>TBHQ</td>
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<td>0.022</td>
<td>0.022</td>
<td>0.022</td>
</tr>
<tr>
<td>Safflower Oil</td>
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<td>45</td>
<td>64.5</td>
<td>70</td>
</tr>
<tr>
<td>Cottonseed Oil</td>
<td>45</td>
<td>43</td>
<td>26</td>
<td>4</td>
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<tr>
<td>Flaxseed Oil</td>
<td>12</td>
<td>22</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Menhaden Oil</td>
<td>-</td>
<td>-</td>
<td>19.5</td>
<td>36</td>
</tr>
<tr>
<td>SFA</td>
<td>18.52</td>
<td>18.25</td>
<td>18.50</td>
<td>17.47</td>
</tr>
<tr>
<td>MUFA</td>
<td>17.58</td>
<td>18.16</td>
<td>17.63</td>
<td>18.07</td>
</tr>
<tr>
<td>PUFA</td>
<td>73.86</td>
<td>73.55</td>
<td>72.42</td>
<td>72.42</td>
</tr>
<tr>
<td>n-6</td>
<td>67.13</td>
<td>61.32</td>
<td>64.97</td>
<td>58.21</td>
</tr>
<tr>
<td>% kcal from n-6</td>
<td>14.8%</td>
<td>13.5%</td>
<td>14.3%</td>
<td>12.8%</td>
</tr>
<tr>
<td>n-3</td>
<td>6.74</td>
<td>12.23</td>
<td>6.42</td>
<td>11.71</td>
</tr>
<tr>
<td>% kcal from n-3</td>
<td>1.5%</td>
<td>2.7%</td>
<td>1.4%</td>
<td>2.6%</td>
</tr>
<tr>
<td>n-6 to n-3</td>
<td>9.97</td>
<td>5.01</td>
<td>10.13</td>
<td>4.97</td>
</tr>
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</table>

Product ID: TD.140812, TD.140813, TD.140814, TD.140815

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1 All diets were developed by Longo AB, Flowers J, and Ward WE (unpublished) and matched for macronutrient content (17.7% protein, 56.5% carbohydrate, 11.2% fat by weight) (17.8% protein, 56.8% carbohydrate, 25.4% fat kcal) and provided 4.0 kcal/g. Total SFA, MUFA, and PUFA were matched across all diets.

2 ALA; alpha-linolenic acid, DHA; docosahexaenoic acid, EPA; eicosapentaenoic acid, MUFA; monounsaturated fatty acid, PUFA; polyunsaturated fatty acid, SFA; saturated fatty acid, TBQH; T-butylhydroquinone.
Sham and OVX Surgery

For Study 3 and 4, rats were randomized to receive Sham and OVX surgery at 3 months of age on-site (Comparative Biosciences Facility, Principal Surgeon: Longo AB). Rats were anesthetized in an induction chamber using isoflurane (rate of delivery between 2-5%) and transitioned to a nose cone for maintenance of the anesthesia (2-5% delivery rate, as needed) and placed on a sterile surgery surface. Metacam (analgesic) and Baytril (antibiotic) were injected subcutaneously prior to surgery. A single dorsal skin incision and bilateral muscle incisions were made and the ovaries were exposed and removed of as much adipose tissue as possible, and removed (OVX). Ovaries were exposed but not removed in the rats randomized to Sham-control surgery. The muscle incisions were closed with dissolvable sutures and the dorsal skin incisions were closed with surgical staples. After surgery, prior to recovery from anesthesia, Buprenorphine (analgesic) and isotonic saline (fluid replacement) were injected subcutaneously. After recovery from anesthesia, rats were returned to a recovery cage and monitored for any complication for four hours post-surgery. Cage-mates had similar surgery-type on the same day and were reunited two days post-op. Rats were weighed and carefully examined for five days after surgery – with additional oral Metacam, injectable Baytril or injectable Buprenorphine being administered daily for up to three days when necessary (one case). Surgical skin staples were removed under light isoflurane anesthesia 7-10 days post-op.

μCT Scanning

For all studies, Sham and OVX rats were initially anesthetized with isoflurane in an induction chamber with a 3-4% flow rate and later transferred to a nose cone with a 1-
2% flow rate for maintenance of general anesthesia. When scanning the hind limb, the leg was held secure with a customizable apparatus (Figure 4-2). In the SkyScan 1176 scanner, the X-ray beam is collimated, restricting radiation to the scan field of view only. Only the scanned leg, held in a polystyrene tube, was included in the field of view and therefore only that leg received exposure to radiation. Rats were maintained under general anesthesia for approximately 25 minutes, which included the time for the induction of anesthesia and the acquisition of the scan. When scanning the proximal tibia in vivo, the field of view began at the knee joint and extended distally 20 mm (Figure 4-3).
Studies 1 through 4 used the above set-up for imaging of the rat hind limb \textit{in vivo}. Study 2 involved scanning of the right and left hind limb.

(Image by Longo AB, published in (233))

A) Raw image and B) reconstructed image of the field of view of the \textit{in vivo} rat hind limb with representative boxes of the region of interest analyzed. Blue box represents ROI analyzed in Study 1 and Study 2. Red box represents region of interest analyzed in Study 3 and Study 4. C) Representative cross-section at which the growth cartilage begins to transition into the proximal tibia.

(Image by Longo AB, unpublished, 2016).
For Study 1 and 2, Sham and OVX rats underwent *in vivo* μCT scanning (SkyScan 1176, Bruker micro-CT, Kontich, Belgium) of the right hind limb at 13, 17, 21, and 25 weeks of age, coinciding with 1, 5, 9, and 13 weeks post-surgery at a voxel size of 18μm. For Study 2, the contralateral hind limb was scanned at 25 weeks of age (13 weeks post-surgery) only, to serve as a non-irradiated internal control at a voxel size of 18μm.

For Study 3, Sham and OVX rats underwent *in vivo* μCT scanning of the right hind limb at 24 weeks of age (12 weeks post-surgery). Within one-week following *in vivo* scanning, rats were euthanized and the right tibia was excised, cleaned of soft tissue, wrapped in saline soaked gauze and stored at -80°C until subsequent scanning. For *ex vivo* scanning of the right proximal tibia, bones were hydrated in phosphate buffered saline (Sigma, St. Louis, MO, USA) at room temperate for two hours and subsequently wrapped in parafilm wax. Parafilm-wrapped tibias were held secure in foam in a plastic tube secured to the scan bed to limit movement. *Ex vivo* tibias were scanned at both 18μm and 9μm voxel sizes.

For Study 4, all rats underwent *in vivo* μCT scanning of the right hind limb at 3 months of age, prior to Sham and OVX surgery at a voxel size of 18μm. At 6 months of age, all rats underwent *in vivo* scanning of the right hind limb and of the abdomen at voxel sizes of 18μm and 35μm, respectively. For *ex vivo* scanning of LV4, bones were hydrated in phosphate buffered saline at room temperature for two hours and subsequently wrapped in parafilm wax. Parafilm-wrapped LV4 were held secure in foam in a plastic tube secured to the scan bed to limit movement. *Ex vivo* LV4 were scanned at 9μm voxel size.
For all studies, scanning parameters were set to limit the scan duration while attaining a high quality image. *In vivo* and *ex vivo* scan parameters used for image acquisition and reconstruction settings (NRecon, v. 1.6.9.10, Bruker micro-CT, Kontich, Belgium) of the proximal tibia, LV, and abdomen (for body composition) are summarized in Table 4-2. The region of interest (ROI) of the tibia to be analyzed began 2.64 mm (Study 1 and 2) or 1.76 mm (Study 3 and 4) distal from the point at which the growth cartilage begins to transition into the proximal tibia metaphysis and extended 3.52 mm toward the ankle (Figure 4-3). The ROI differed between Studies 1 and 2 and Studies 3 and 4 because of the drastic loss of trabecular bone that occurred 3-months post-OVX. The ROI of the abdomen to be analyzed for body composition extended the length of one vertebral body (LV3).
Table 4-2. Scan, reconstruction, and analysis parameters by µCT

<table>
<thead>
<tr>
<th>Scanning Parameters</th>
<th>Tibia and/or LV4</th>
<th>Abdomen</th>
</tr>
</thead>
<tbody>
<tr>
<td>18µm in vivo</td>
<td>18µm ex vivo</td>
<td>9µm ex vivo</td>
</tr>
<tr>
<td>Voxel Size (µm)</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Voltage (kV)</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>Amperage (µA)</td>
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<table>
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<tr>
<th>Reconstruction Parameters</th>
<th>Tibia and/or LV4</th>
<th>Abdomen</th>
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<tr>
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<td>Gaussian</td>
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<td>Smoothing Kernel</td>
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<tr>
<td>Ring Artifact Reduction</td>
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<td>8</td>
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<td>Beam Hardening Correction (%)</td>
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<td>30</td>
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<th>Analysis Parameters</th>
<th>Tibia and/or LV4</th>
<th>Abdomen</th>
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<tr>
<td>Thresholding</td>
<td>Global, 102</td>
<td>Global, 102</td>
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<tr>
<td>Trabecular Thresholding</td>
<td>Adaptive, 76</td>
<td>Adaptive, 76</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1 All µCT scans were obtained using the SkyScan 1176 model, Bruker micro-CT, Kontich, Belgium.
The distinction between trabecular and cortical bone within the ROI of scans of the proximal tibia were defined and separated by automatic processes (created by Longo AB) and analyzed separately (CT Analyzer, v. 1.14.4.1 + (64-bit), SkyScan, Bruker micro-CT, Kontich, Belgium) (Figure 4-4). In brief, bone tissue was identified by a global thresholding procedure followed by a series of morphological operations to separate the trabecular processes from the cortical shell. Once segmented, trabecular and cortical regions were defined and saved as distinct ROIs. Trabecular and cortical bone were binarized using an adaptive and global thresholding procedure, respectively. The resulting ROIs were subsequently analyzed. The trabecular microarchitecture measurements, including BMD, BV/TV, connectivity density (Conn.D), degree of anisotropy (DA), Tb.Th., Tb.N. and Tb.Sp. were quantified by 3D analysis of the defined ROI. The cortical microarchitecture measurements, including BMD, cross-sectional area inside the periosteal envelope (Tt.Ar.), Ct.Ar., Ct.Ar./Tt.Ar., Ct.Th., Ps.Pm., Ec.Pm., Ma.Ar., and mean eccentricity (Ecc.) were performed using 2D analysis of the defined ROI. A summary of the microarchitecture measurements assessed is outlined in Table 4-3. A summary and general description of the primary and secondary trabecular and cortical microarchitectural outcomes are reported in Table 4-4.

Non-lean mass, lean mass, and bone mass within the ROI of scans of the abdomen were defined and separated by global thresholding. Thresholding values of 1-34, 35-93, and 94-255 were set for non-lean mass, lean mass, and bone mass, respectively. The percent object volume (Obj.V/TV) was assessed by 2D analysis of the defined ROI (CT Analyzer, v. 1.14.4.1 + (64-bit), SkyScan, Bruker micro-CT, Kontich, Belgium) and made relative to the ROI length.
Figure 4-4. Representative (A) cross-section of proximal tibia with (B) cortical and (C) trabecular bone defined and separated by automatic processes.

Through a series of thresholding and morphological operations, the binarized B) cortical and C) trabecular bone can be defined, separated and analyzed. The same process can be applied to all samples to automatically segment bone compartments without bias.

(Image by Longo AB, published in (234))
<table>
<thead>
<tr>
<th>Trabecular Outcomes</th>
<th>General Implication of a Higher Value for Bone Health</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BMD (g/cm³)</strong></td>
<td>Volumetric mineral density per unit volume Positive</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>Percentage of area within the ROI occupied by bone Positive</td>
</tr>
<tr>
<td>Conn.D (mm⁻³)</td>
<td>Number of redundant connections between trabecular structures per unit volume Positive</td>
</tr>
<tr>
<td></td>
<td>Orientation of the trabecular network, 1= isotropic (lacking orientation), &gt;1= anisotropic (highly oriented)</td>
</tr>
<tr>
<td>DA (no unit)</td>
<td>Positive</td>
</tr>
<tr>
<td>Tb.Th. (mm)</td>
<td>Average thickness of the trabeculae Positive</td>
</tr>
<tr>
<td>Tb.N. (mm⁻¹)</td>
<td>Average number of trabeculae per unit length Positive</td>
</tr>
<tr>
<td>Tb.Sp. (mm)</td>
<td>Average distance between trabeculae assessed by ‘3D sphere-fitting’ Negative</td>
</tr>
<tr>
<td><strong>Cortical Outcomes</strong></td>
<td></td>
</tr>
<tr>
<td>Tt.Ar. (mm²)</td>
<td>Cross-sectional area within the outer perimeter of bone (periosteum) Positive</td>
</tr>
<tr>
<td>Ct.Ar. (mm²)</td>
<td>Area of the cortical bone only Positive</td>
</tr>
<tr>
<td>Ct.Ar./Tt.Ar. (%)</td>
<td>Percentage of area within the ROI occupied by bone Positive</td>
</tr>
<tr>
<td>Ct.Th. (mm)</td>
<td>Average thickness of the cortical bone Positive</td>
</tr>
<tr>
<td>Ps.Pm. (mm)</td>
<td>Perimeter of the outer bone (periosteum)</td>
</tr>
<tr>
<td>Ec.Pm. (mm)</td>
<td>Perimeter of the inner bone (endosteum)</td>
</tr>
<tr>
<td>Ma.Ar. (mm²)</td>
<td>Area of the medullary cavity</td>
</tr>
<tr>
<td>Ecc.</td>
<td>Circular shape of bone, 0= circle, &gt;0= toward ellipsoid</td>
</tr>
<tr>
<td><strong>Body Composition Outcome</strong></td>
<td></td>
</tr>
<tr>
<td>Obj.V/TV (%)</td>
<td>Percentage of area within the ROI occupied by binarized object (predefined by thresholding values)</td>
</tr>
</tbody>
</table>

1 BMD; bone mineral density, BV/TV, percent bone volume, Conn.D; connectivity density, Ct.Ar.; cortical area, Ct.Ar./Tt.Ar.; cortical area fraction, Ct.Th.; cortical thickness, DA; degree of anisotropy, Ec.Pm.; endocortical perimeter, Ecc.; mean eccentricity, Ma.Ar.; medullary area, Obj.V./TV; object area fraction, Ps.Pm.; periosteal perimeter, Tb.N.; trabecular number, Tb.Sp.; trabecular separation, Tb.Th.; trabecular thickness, Tt.Ar.; total bone area.

2 Standardized nomenclature and units are derived from (61, 235).

3 Bolded outcomes are primary outcomes. Non-bolded outcomes are secondary outcomes.
Serum Biochemical Markers

To assess serum markers of bone cell activity, serum OPG and RANKL were quantified using a Milliplex Rat OPG-Single Plex (RBN1MAG-31K-01, Millipore Canada, Toronto, Ont) and a Milliplex Rat RANKL-Single Plex (RRNKLMAG-31K-01, Millipore Canada, Toronto, Ont) according to the manufacturer’s instructions. Data was collected using the MagPix (Luminex xMAP, Toronto, Ont). The standard curve ranges from 10-40,000 pg/mL for OPG and 3.7-15,000 pg/mL for RANKL. The minimum detection limit is 4.4 pg/mL for OPG and 2.6 pg/mL for RANKL. All values were quantified in triplicate and determined by comparison to a standard curve prepared by the supplier.

Statistical Analyses

Study 1: The effects of hormone status (with two levels: Sham and OVX), and time, and their interaction for each dependent variable (body weight, food intake) was investigated using a two-way repeated measures ANOVA. Time was assessed on 13 levels for analysis of weekly body weights and on 26 levels for analysis of twice weekly food intakes. Where a significant interaction was found, a Bonferroni post hoc test was used to test the main effects between Sham and OVX rats at each time point.

A two-way repeated measures ANOVA was used to determine an effect of time post-surgery (13, 17, 21, and 25 weeks) and hormone status (Sham versus OVX) on longitudinal microarchitecture measures of the right proximal tibia. Where a significant interaction was found, a Bonferroni post hoc test was used to test the main effects between Sham and OVX rats at each time point. In the event of missing values, the series mean was used to replace those missing values. Missing values resulted from rat
movement during the scan that caused poor image quality, not allowing for adequate analysis.

**Study 2:** To determine an effect of irradiation, a two-way ANOVA was used to compare the repeatedly irradiated right tibia to the non-irradiated left tibia with hormone status (Sham versus OVX) and irradiation frequency (right hind limb versus left hind limb) as independent factors at 25 weeks of age.

**Study 3:** A two-tailed independent t-test was used to determine differences in final body weights, final food intake, wet uterine weights, and microarchitecture outcomes between Sham and OVX rats. The effect of scanning protocols (9µm ex vivo, 18µm ex vivo, 18µm in vivo) for all trabecular and cortical morphological outcomes was assessed using a one-way ANOVA. A Bonferroni post hoc test was used to compare differences between groups. A Pearson’s correlation will be used to assess correlations between the microarchitecture outcomes calculated by 9µm ex vivo (independent variable) and 18µm ex vivo and 18µm in vivo (dependent variables) scanning protocols. It is well established that OVX has a drastic and deleterious effect to bone morphology at the proximal tibia. For this reason, bone outcomes for Sham and OVX rats were further analyzed separately, so that the effect or interaction of hormone status did not overshadow any more subtle effects of scanning parameters. A power analysis of each scanning protocol was performed retrospectively to determine the number of animals needed to identify differences in the primary microarchitecture outcomes between Sham and OVX groups. Power was set at 0.80 and type I error rate was set at 0.05 for all power analyses.
**Study 4:** A one-way repeated measures ANOVA was used to determine the effect of diet on body weight and food intake from 1 through 3 months of age. A two-way repeated measures ANOVA was used to determine the effect of diet, hormone status and their interaction on body weight and food intake from 3 through 6 months of age. A Bonferroni post hoc test was used to test the main effects of diet at each time point when a significant interaction was found.

A one-way ANOVA was used to determine the effect of diet on bone microarchitecture outcomes (proximal tibia and LV4) from 1 through 3 months of age. A two-way ANOVA was used to determine the effect of diet, hormone status, and their interaction on uterine weights and bone outcomes at 6 months of age (proximal tibia, LV4, serum biomarkers). Because changes in bone structure of the proximal tibia occurred between 1 through 3 months of age and because the same rat was followed longitudinally, the absolute difference in microarchitecture outcomes of the proximal tibia from 3 through 6 months of age was assessed using a two-way ANOVA. This secondary analysis allowed for an investigation into the contribution of diet on the bone microarchitecture of the proximal tibia following Sham or OVX surgery.

Because of the strong effect of hormone status, bone microarchitecture outcomes for Sham and OVX groups at 6 months of age and the longitudinal change were analyzed independently within each hormone status group (Sham, OVX) by one-way ANOVA. This analysis was done to remove the large influence of hormone status and to allow any subtle effects of diet to be observed within each hormone status group. A Bonferroni post hoc test was used to test the main effects of diet and/or hormone status when a significant interaction was found.
All statistical analyses were performed using SPSS Statistics (v. 22, IBM). The level of significance was set at $p<0.05$ for all analyses. All food intake and body weight data are presented as Mean $\pm$ SEM. All uterine weights, $\mu$CT data and serum biomarker data are presented as Mean $\pm$ SD.
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Chapter 5: Study 1 and 2

Longitudinal Use of μCT does not Alter Microarchitecture of the Proximal Tibia in Sham or OVX SD Rats

Amanda B. Longo, Sandra M. Sacco, Phil L. Salmon, and Wendy E. Ward

Abstract

In vivo µCT provides the ability to measure longitudinal changes to tibia microarchitecture, but the effect of this radiation is not well understood. The right proximal tibia of SD rats (n=12/group) randomized to Sham-control or OVX surgery at 12 weeks of age was scanned using µCT at 13, 17, 21, and 25 weeks of age, at a resolution of 18µm and a radiation dose of 603 mGy. The left proximal tibia was scanned only at 25 weeks of age to serve as an internal non-irradiated control. Repeated irradiation did not affect tibia microarchitecture in Sham or OVX groups, although there was an increase in cortical Ecc. (P<0.05). All trabecular outcomes and cortical BMD were different (P<0.05) between groups after only 1 week post-surgery and differences persisted to study endpoint. Characteristic changes to trabecular bone were observed in OVX rats over time. Interactions of time and hormone status were found for cortical BMD (P<0.001), Ps.Pm., and Ec.Pm. (P<0.05). Repeated irradiation of the tibia at 13, 17, 21, and 25 weeks of age does not cause adverse effects to microarchitecture, regardless of hormone status. This radiation dose can be applied over a typical 3-month study period to comprehensively understand how an intervention alters tibia microarchitecture without confounding effects of radiation.

Introduction

µCT imaging is the gold standard for the direct 3-dimensional modeling and assessment of the trabecular and cortical microarchitecture in pre-clinical animal studies (2-4). Of more recent interest is the ability to measure changes in vivo to bone microarchitecture at multiple time points throughout the lifespan in response to an
intervention. Because \( \mu \)CT imaging is a non-destructive method, its use may substantively reduce the number of animals required for longitudinal testing of an outcome and allows for repeated measurement of the same animal, which may minimize the impact of inter-animal variability that may arise with a cross-sectional study design.

Although the data obtained from repeated \( \mu \)CT scanning may provide powerful evidence for a change in bone health in response to an intervention within the same animal, the effect of exposure to radiation may be detrimental to the very region under investigation (5-7). Some studies investigating the effect of radiation exposure by \( \mu \)CT to the hind limb or rats suggest a resilience to 500 or 900 mGy radiation, respectively (6, 8), although one study of adult rats found a difference between irradiated and non-irradiated limbs after exposure to 400 mGy of radiation, repeated 5 times (5). In a male mouse model, three repeated radiation doses of 776 mGy caused significant reductions in trabecular bone volume and Tb.N., while a similar scanning protocol applying 434 mGy caused no damage to the irradiated tibia (7). High-radiation dose scans produce high-quality images, but a balance must be struck between radiation exposure and images of sufficient quality to be analyzed. While the skeletal site-specific loss of bone mineral and microarchitectural integrity after OVX is well characterized (9), whether hormone status modulates the skeleton’s susceptibility to radiation-induced damage is less well understood.

Because the OVX rat model is the most widely used pre-clinical model of osteoporosis and has been used extensively for developing prevention and treatment strategies (10, 11), the primary objective of this study was to investigate the effect of repeated irradiation to bone structure over a typical 12-week protocol by comparing the
irradiated and non-irradiated limb. Although previous studies have investigated the effects of irradiation dose between 400 and 900 mGy per scan at intervals between 1 and 20 weeks (5-8), this study used specific parameters that resulted in a low signal-to-noise ratio and high-quality images with a total absorbed dose of 2400 mGy delivered locally over 12 weeks. Twelve weeks is a typical time period in which the response of an intervention is studied using the OVX rat model. The average remodeling period in the rat and human is roughly 40 and 200 days, respectively (12, 13). Therefore, a 12-week trial in the OVX rat results in approximately two complete remodeling cycles; this is roughly equivalent to a one to one-and-a-half year clinical trial in humans. Also, the early increase in bone turnover favouring bone resorption as reported in postmenopausal women is mimicked in the first 14 days post-OVX in the rat model (12, 14). Because no study to date has followed the same rat over a 12-week study period following OVX at 12 weeks of age, to determine changes in both the trabecular and cortical bone using µCT, a secondary aim is to investigate the longitudinal effect of hormonal status on trabecular and cortical bone of the proximal tibia to further characterize this pre-clinical model of postmenopausal osteoporosis.

Materials and Methods

*See Chapter Four – Experimental Approach for methodologies regarding Animals and Diet, µCT, and Statistical Analyses

Radiation Dose

Radiation dose was calculated using a portable dosimeter on high bias setting scanned in air (TN-502RD-H, Best Medical Canada, Ottawa, Canada). The probe was secured in the center of the field of view and was scanned under the same parameters as
the rat hind limbs. Radiation dose was calculated as an average of five scans over the five days that scanning occurred. Since the rats were scanned *in vivo*, the layer of soft tissue surrounding the bone would have attenuated some of the radiation. Thus, the calculated radiation dose may slightly overestimate the actual X-ray exposure to bone.

**Results**

*Body Weight and Food Intake*

A significant interaction (P<0.05) and a significant main effect of hormone status and age (P<0.001) were observed for body weight. One week post-surgery, OVX rats had a greater body weight than Sham rats and this persisted until study endpoint, age 25 weeks (P<0.05). At study endpoint, average body weight of Sham and OVX rats was 343.1 ± 28.7 g and 387.5 ± 38.1 g, respectively (*Figure 5-1*). A significant interaction (P<0.05) and a significant main effect of hormone status and age (P<0.001) were observed for food intake. Food intake did not differ between Sham and OVX rats between 1 and 2 weeks post-surgery. Food intake was greater (P<0.05) in OVX rats compared to Sham rats from week 2.5 through week 6 post-surgery. Food intake did not differ between treatment groups from 6.5 weeks post-surgery to study endpoint, age 25 weeks (*Figure 5-2*).
Figure 5-1. Weekly body weight of Sham and OVX rats from 1 to 13 weeks post-surgery.

Values are Mean ± SEM, n=12/group. Differences from Sham at a time point are denoted by * (P<0.05) and † (P<0.001).
Figure 5-2. Weekly food intake of Sham and OVX rats from 1 to 13 weeks post-surgery.

Values are Mean ± SEM, n=12/group. Differences from Sham at a time point are denoted by * (P<0.05).
Radiation Dose

The average radiation dose for one scan in air was 603 ± 8.3 mGy.

Radiation Effect on Bone Microarchitecture

The effect of radiation exposure was determined by comparing the repeatedly irradiated right proximal tibia to the singly irradiated left proximal tibia at 25 weeks of age (13 weeks post-surgery). No interactions were observed between irradiation and hormone status for any of the trabecular outcomes measured when the right and left proximal tibia were compared (Table 5-1). No independent effect of irradiation was observed for any trabecular outcome. As expected, OVX resulted in a decrease in BMD, BV/TV, and Tb.N. and an increase in Tb.Sp. and DA (P<0.001) compared to Sham rats at study endpoint. No difference in Tb.Th. was observed between groups.

No interactions were observed between irradiation and hormone status for any cortical outcomes examined (Table 5-2). Radiation exposure resulted in an increased cortical Ecc. of the proximal tibia (P<0.05). OVX caused a decrease in cortical BMD and Ec.Pm. (P<0.001) and an increase in Ps.Pm. (P<0.05) compared to Sham rats at study endpoint.
Table 5-1. Effect of irradiation to trabecular microarchitecture of the proximal tibia in 25-week old Sham and OVX rats\textsuperscript{1,2}

<table>
<thead>
<tr>
<th>Trabecular Outcomes</th>
<th>Sham Non-irradiated, Left proximal tibia</th>
<th>Sham Irradiated, Right proximal tibia\textsuperscript{a}</th>
<th>OVX Non-irradiated, Left proximal tibia</th>
<th>OVX Irradiated, Right proximal tibia</th>
<th>Irradiation X Hormone Status</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD (g/cm\textsuperscript{3})</td>
<td>0.258 ± 0.091</td>
<td>0.255 ± 0.098</td>
<td>0.033 ± 0.018</td>
<td>0.026 ± 0.018</td>
<td>0.920</td>
<td>0.796</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>24.87 ± 5.01</td>
<td>24.69 ± 5.38</td>
<td>2.58 ± 1.24</td>
<td>2.08 ± 1.16</td>
<td>0.886</td>
<td>0.761</td>
</tr>
<tr>
<td>Tb.Th. (mm)</td>
<td>0.083 ± 0.005</td>
<td>0.083 ± 0.008</td>
<td>0.083 ± 0.011</td>
<td>0.079 ± 0.008</td>
<td>0.363</td>
<td>0.370</td>
</tr>
<tr>
<td>Tb.N. (mm\textsuperscript{1})</td>
<td>2.992 ± 0.521</td>
<td>2.994 ± 0.690</td>
<td>0.317 ± 0.166</td>
<td>0.260 ± 0.157</td>
<td>0.835</td>
<td>0.849</td>
</tr>
<tr>
<td>Tb.Sp. (mm)</td>
<td>0.229 ± 0.048</td>
<td>0.253 ± 0.081</td>
<td>1.394 ± 0.240</td>
<td>1.465 ± 0.266</td>
<td>0.663</td>
<td>0.372</td>
</tr>
<tr>
<td>DA</td>
<td>2.065 ± 0.160</td>
<td>2.063 ± 0.140</td>
<td>2.450 ± 0.470</td>
<td>2.198 ± 0.477</td>
<td>0.467</td>
<td>0.446</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Values are expressed as Mean ± SD of rats at 25 weeks of age (13 weeks post-surgery) after having undergone no irradiation (left proximal tibia) compared to repeated irradiation (right proximal tibia), n=12/group

\textsuperscript{a} Where one missing value occurred, the values were replaced by the series mean. Due to animal movement during scanning, trabecular microarchitecture could not be obtained for this sample.

\textsuperscript{2} BMD; bone mineral density, BV/TV; percent bone volume, DA; degree of anisotropy, Tb.N.; trabecular number, Tb.Sp.; trabecular separation, Tb.Th.; trabecular thickness.
Table 5-2. Effect of irradiation to cortical microarchitecture of the proximal tibia in 25-week old Sham and OVX rats$^{1,2}$

<table>
<thead>
<tr>
<th>Cortical Outcomes</th>
<th>Sham</th>
<th>OVX</th>
<th>Irradiation X Hormone Status</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-irradiated, Left proximal tibia</td>
<td>Irradiated, Right proximal tibia</td>
<td>Non-irradiated, Left proximal tibia</td>
<td>Irradiated, Right proximal tibia</td>
</tr>
<tr>
<td>BMD (g/cm$^3$)</td>
<td>1.388 ± 0.006</td>
<td>1.389 ± 0.005</td>
<td>1.372 ± 0.003</td>
<td>1.372 ± 0.003</td>
</tr>
<tr>
<td>Tt.Ar. (mm$^2$)</td>
<td>12.164 ± 0.978</td>
<td>11.833 ± 0.936</td>
<td>12.076 ± 0.662</td>
<td>12.095 ± 0.877</td>
</tr>
<tr>
<td>Ct.Ar. (mm$^2$)</td>
<td>6.375 ± 0.557</td>
<td>6.530 ± 0.511</td>
<td>6.410 ± 0.231</td>
<td>6.442 ± 0.240</td>
</tr>
<tr>
<td>Ct.Ar./Tt.Ar. (%)</td>
<td>52.53 ± 4.06</td>
<td>52.53 ± 4.06</td>
<td>53.44 ± 3.24</td>
<td>53.17 ± 2.24</td>
</tr>
<tr>
<td>Ct.Th. (mm)</td>
<td>0.465 ± 0.038</td>
<td>0.484 ± 0.026</td>
<td>0.467 ± 0.015</td>
<td>0.475 ± 0.018</td>
</tr>
<tr>
<td>Ps.Pm. (mm)</td>
<td>26.195 ± 1.228</td>
<td>25.715 ± 1.087</td>
<td>26.851 ± 0.896</td>
<td>26.548 ± 0.820</td>
</tr>
<tr>
<td>Ec.Pm. (mm)</td>
<td>1.257 ± 0.303</td>
<td>1.254 ± 0.379</td>
<td>0.630 ± 0.136</td>
<td>0.581 ± 0.114</td>
</tr>
<tr>
<td>Ma.Ar. (mm$^2$)</td>
<td>5.788 ± 0.775</td>
<td>5.304 ± 0.595</td>
<td>5.666 ± 0.535</td>
<td>5.653 ± 0.793</td>
</tr>
<tr>
<td>Ecc.</td>
<td>0.461 ± 0.051</td>
<td>0.522 ± 0.045</td>
<td>0.473 ± 0.078</td>
<td>0.525 ± 0.074</td>
</tr>
</tbody>
</table>

$^1$ Values are expressed as Mean ± SD of rats at 25 weeks of age (13 weeks post-surgery) after having undergone no irradiation (left proximal tibia) compared to repeated irradiation (right proximal tibia), n=12/group.

$^a$ Where one missing value occurred, the values were replaced by the series mean. Due to animal movement during scanning, cortical microarchitecture could not be obtained for these samples.

$^2$ BMD; bone mineral density, Ct.Ar.; cortical bone area, Ct.Ar./Tt.Ar.; cortical area fraction, Ct.Th.; cortical thickness, Ec.Pm.; endocortical perimeter, Ecc.; mean eccentricity, Ma.Ar.; medullary area, OVX; ovariectomized, Ps.Pm.; periosteum perimeter, Tt.Ar.; cross sectional area inside the periosteal envelope.
Longitudinal Effect on Trabecular Bone Microarchitecture

One week post-surgery, coinciding with 13 weeks of age, all trabecular outcomes except DA were significantly different between Sham and OVX, and these differences were maintained throughout the study period in all but Tb.Th., which returned to similar values by 21 weeks of age (Table 5-3). When the right proximal tibia of Sham or OVX rats was scanned at 4-monthly intervals between ages of 13 and 25 weeks, an interaction was observed between age and the hormone status for BV/TV (P<0.05), Tb.N. (P<0.001), and Tb.Sp. (P<0.001). No interaction was observed for BMD (P=0.11), DA (P=0.079), or Tb.Th. (P=0.19). An effect of age was observed for BMD, DA, and Tb.Th. (P<0.05), and an effect of hormone status was observed for BMD (P<0.001).

Sham rats showed no significant changes to trabecular BMD, BV/TV, or Tb.Sp. with time. A decline in Tb.Th. (P<0.05) from 13 to 17 weeks of age and increase (P<0.05) from 17 to 25 weeks of age coincided with a decrease (P<0.05) from 17 to 21 weeks of age for Tb.N. in Sham rats. Trabecular BMD, BV/TV, Tb.N., and Tb.Sp. all showed a similar pattern over time in OVX rats where significant differences were observed among all time points except for between 21 and 25 weeks of age. OVX rats experienced a decline (P<0.05) in Tb.Th. between ages of 13 and 17 weeks followed by an increase (P<0.001) in Tb.Th. from age 21 to 25 weeks. Both Sham and OVX rats experienced a significant reduction in DA between 13 and 17 weeks of age (P<0.05).
Table 5.3. Longitudinal changes to trabecular microarchitecture of the right proximal tibia in Sham and OVX rats at 13, 17, 21, and 25 weeks of age.1,2

<table>
<thead>
<tr>
<th>Age (week)</th>
<th>13</th>
<th>17</th>
<th>21</th>
<th>25</th>
<th>13</th>
<th>17</th>
<th>21</th>
<th>25</th>
<th>Age X Hormone Status</th>
<th>Age</th>
<th>Hormone Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time Post-</td>
<td>1</td>
<td>5</td>
<td>9</td>
<td>13</td>
<td>1</td>
<td>5</td>
<td>9</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgery (week)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trabecular Outcomes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD (g/cm³)</td>
<td>0.279±0.100</td>
<td>0.269±0.095</td>
<td>0.244±0.107</td>
<td>0.255±0.098</td>
<td>0.155±0.097</td>
<td>0.082±0.046</td>
<td>0.035±0.025</td>
<td>0.026±0.017</td>
<td>0.110</td>
<td>0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>26.00±5.50</td>
<td>25.43±5.22</td>
<td>24.07±5.91</td>
<td>24.69±5.38</td>
<td>17.70±6.64</td>
<td>5.88±3.21</td>
<td>2.66±1.70</td>
<td>2.08±1.17</td>
<td>0.002</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tb.Th. (mm)</td>
<td>0.080±0.004</td>
<td>0.076±0.004</td>
<td>0.081±0.007</td>
<td>0.083±0.008</td>
<td>0.076±0.004</td>
<td>0.072±0.005</td>
<td>0.083±0.006</td>
<td>0.079±0.008</td>
<td>0.190</td>
<td>0.007</td>
<td>0.093</td>
</tr>
<tr>
<td>Tb.N. (mm⁻¹)</td>
<td>3.254±0.713</td>
<td>3.330±0.640</td>
<td>2.991±0.693</td>
<td>2.994±0.690</td>
<td>2.319±0.853</td>
<td>0.810±0.401</td>
<td>0.325±0.215</td>
<td>0.266±0.156</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tb.Sp. (mm)</td>
<td>0.230±0.074</td>
<td>0.245±0.077</td>
<td>0.264±0.095</td>
<td>0.253±0.081</td>
<td>0.360±0.167</td>
<td>1.099±0.339</td>
<td>1.420±0.306</td>
<td>1.465±0.266</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DA</td>
<td>2.397±0.196</td>
<td>2.093±0.151</td>
<td>1.997±0.199</td>
<td>2.063±0.140</td>
<td>2.358±0.121</td>
<td>2.098±0.209</td>
<td>2.289±0.244</td>
<td>2.198±0.477</td>
<td>0.079</td>
<td>0.003</td>
<td>0.093</td>
</tr>
</tbody>
</table>

1 Values are expressed as Mean ± SD, n=12/group. Different letters denote a significant difference within treatment over time (P<0.05). Significant differences from Sham within a time point are shown by * P (<0.05) or † (P<0.001).

Where one or two missing values occurred, the values were replaced with the series mean. Due to animal movement during scanning, trabecular microarchitecture could not be obtained for these samples.

2 BMD; bone mineral density, BV/TV; percent bone volume, DA; degree of anisotropy, Tb.N.; trabecular number, Tb.Sp.; trabecular separation, Tb.Th.; trabecular thickness.
Table 5-4. Longitudinal changes to cortical microarchitecture of the right proximal tibia in Sham and OVX rats at 13, 17, 21, and 25 weeks of age \(^1,2\)

<table>
<thead>
<tr>
<th>Cortical Outcomes</th>
<th>BMD (g/cm(^3))</th>
<th>Tt.Ar. (mm(^2))</th>
<th>Ct.Ar. (mm(^2))</th>
<th>Ct.Ar./Tt.Ar. (%)</th>
<th>Ps.Pm. (mm)</th>
<th>Ec.Pm. (mm)</th>
<th>Ma.Ar. (mm(^2))</th>
<th>Ecc.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sham</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (week) 13</td>
<td>1.385 ± 0.004</td>
<td>11.953 ± 0.909a</td>
<td>0.369 ± 0.369a</td>
<td>47.34 ± 3.02a</td>
<td>26.580 ± b</td>
<td>1.431 ± ab</td>
<td>6.310 ± 0.747a</td>
<td>0.547 ± 0.033a</td>
</tr>
<tr>
<td>17(^e)</td>
<td>1.390 ± 0.003</td>
<td>12.412 ± 0.879a</td>
<td>0.263 ± 0.263b</td>
<td>51.32 ± 2.50b</td>
<td>26.607 ± b</td>
<td>1.590 ± a</td>
<td>6.062 ± 0.702a</td>
<td>0.528 ± 0.036ab</td>
</tr>
<tr>
<td>21</td>
<td>1.389 ± 0.004</td>
<td>12.021 ± 0.971bc</td>
<td>0.361 ± 0.361c</td>
<td>54.58 ± 2.81c</td>
<td>26.032 ± ab</td>
<td>1.466 ± 0.418a</td>
<td>5.480 ± 0.727b</td>
<td>0.521 ± 0.042b</td>
</tr>
<tr>
<td>25(^d)</td>
<td>1.390 ± 0.005</td>
<td>11.834 ± 0.936bc</td>
<td>0.511 ± 0.511bc</td>
<td>54.55 ± 4.08c</td>
<td>25.715 ± c</td>
<td>1.245 ± 0.695ab</td>
<td>5.304 ± 0.595b</td>
<td>0.522 ± 0.045ab</td>
</tr>
<tr>
<td><strong>OVX</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (week) 13</td>
<td>1.378 ± 0.004a</td>
<td>12.162 ± 1.222ac</td>
<td>0.236 ± 0.236a</td>
<td>46.06 ± 4.32a</td>
<td>26.859 ± a</td>
<td>1.251 ± 0.223a</td>
<td>6.350 ± 0.236a</td>
<td>0.431 ± 0.020a</td>
</tr>
<tr>
<td>17(^c)</td>
<td>1.373 ± 0.004b</td>
<td>12.976 ± 1.062b</td>
<td>0.259 ± 0.259b</td>
<td>48.20 ± 3.92ab</td>
<td>27.956 ± ab</td>
<td>0.925 ± 0.203b</td>
<td>6.540 ± 0.259b</td>
<td>0.462 ± 0.026ab</td>
</tr>
<tr>
<td>21</td>
<td>1.372 ± 0.004b</td>
<td>12.362 ± 0.895a</td>
<td>0.245 ± 0.245bc</td>
<td>51.66 ± 3.52ab</td>
<td>26.914 ± ab</td>
<td>0.659 ± 0.123bc</td>
<td>6.362 ± 0.895b</td>
<td>0.475 ± 0.026bc</td>
</tr>
<tr>
<td>25</td>
<td>1.372 ± 0.003b</td>
<td>12.195 ± 0.877c</td>
<td>0.240 ± 0.240c</td>
<td>53.44 ± 3.24c</td>
<td>26.548 ± d</td>
<td>0.581 ± 0.114cf</td>
<td>6.442 ± 0.877c</td>
<td>0.475 ± 0.026bc</td>
</tr>
</tbody>
</table>

\(^1\) Values are expressed as Mean ± SD, n=12/group. Different letters denote a significant difference within treatment over time (P<0.05). Significant differences from Sham within a time point are shown by * P (<0.05) or † (P<0.001).
Where one or two missing values occurred, the values were replaced with the series mean. Due to animal movement during scanning, trabecular microarchitecture could not be obtained for these samples.

BMD; bone mineral density, Ct.Ar.; cortical bone area, Ct.Ar./Tt.Ar.; cortical area fraction, Ct.Th.; cortical thickness, Ec.Pm.; endocortical perimeter, Ecc.; mean eccentricity, Ma.Ar.; medullary area, OVX; ovariectomized, Ps.Pm.; periosteum perimeter, Tt.Ar.; cross sectional area inside the periosteal envelope.
Discussion

Our findings show that µCT scanning of the proximal tibia at monthly intervals over a standard 3-month time period following surgery, Sham or OVX, does not alter the main parameters of microarchitecture of the tibia. Lack of differences between any of the major 3D outcomes of either trabecular or cortical bone for the right leg (underwent 4 scans) or left leg (only scanned at study endpoint) suggests that researchers can apply this radiation dose at monthly intervals over a typical 3-month study period to comprehensively understand how a diet or other intervention impacts both trabecular and cortical bones of the rat tibia without the confounding effects of radiation. This evidence supports the resilience of the long bones of the rat to repeated irradiation at an absorbed dose of 600 mGy, and that this resilience is independent of hormone status.

The only main effect of radiation exposure was a decrease in mean Ecc., an outcome that quantifies the deviation of a bone’s shape from circular toward elliptical. Ecc. describes the asymmetric distribution of a bone and is associated with aging and OVX in mice and rats, respectively (15, 16). Changes to cortical eccentricity have often been thought to occur in response to the loads applied to bones, but since the current study found differences between contralateral limbs, altered weight bearing cannot be the cause. It is possible that this finding is a false positive due to chance, which becomes more likely as the number of multiple statistical comparisons being made increases.

Our findings are supportive of previous work, which found no effect of either weekly or bi-weekly scanning at radiation doses of approximately 900 or 600 mGy, respectively (6, 8). In these studies, the rats used were roughly 8-month retired breeder Wistar, older than those used in the current study. With increasing age, the rate of bone
turnover changes in favour of bone resorption (9-11, 14, 17-24). Therefore, it is possible that radiation exposure may have less of an impact to bone tissue with increasing age, as bone modeling rates slow. In fact, the only study citing radiation-induced changes to skeletally mature (10 month + 54 week study period) ovary-intact Wistar rats having undergone five scans of 400 mGy compared to their age-matched non-irradiated controls found a decrease in structure model index and an increase in Tb.Th. (5). In this study, the non-irradiated controls were scanned only at study endpoint, which introduces a source of variation that the current study was able to eliminate by comparing contralateral limbs. Similar studies have been conducted using an ovary intact mouse model and have found a radiation dose response whereby repeated irradiation (776 mGy) caused changes to the trabecular microarchitecture of the tibia but lower doses of radiation (166 and 434 mGy) did not (7).

The time points in our study were chosen to mimic those commonly used in OVX rat studies of a nutritional intervention (25-32) so that the data may be applicable for the effective design of future experiments. OVX has been shown to cause skeletal site-specific effects to bone health (9, 14, 15, 33). Overall, the proximal tibia is considered to show the earliest and the greatest changes to trabecular bone mineral and microarchitecture (9), and so this is often the primary site of investigation in studies of surgically induced compromised bone health (5, 23, 34-37).

There was a significant interaction between hormone status and the age of the rats for the trabecular outcomes BV/TV, Tb.N., and Tb.Sp. This interaction suggests that the effect of time on microarchitecture outcomes was different between Sham and OVX groups. Although both Sham and OVX groups experienced statistically significant
differences between some of the microarchitecture outcomes within interim time points, these findings are likely due to random variation associated with bone remodeling at these ages. As expected, over the 12-week study period, the OVX group experienced the greatest reduction in BV/TV (-88.25 %) and Tb.N. (-88.26 %) and the greatest increase in Tb.Sp. (+308.33 %) compared to their Sham counterparts. While Sham rats experienced the same pattern, the magnitude of change was much smaller. Although comparable to some studies (38, 39), the current findings suggest more dramatic trabecular bone loss than what has been found in studies of older Wistar rats (40, 41). Our findings are supportive of a disruption of normal bone modeling with OVX at 3 months of age causing a premature switch to remodeling and loss of trabecular BV/TV.

The finding that Tb.Th. was unaffected by hormone status suggests that the decrease in BV/TV observed with time in the OVX group is due to a decrease in the total number of trabeculae and greater separation of those remaining trabeculae, rather than the overall reduction and thinning of the entire trabecular framework. Waarsing and colleagues (5) have speculated that the trabecular structures neighbouring individual trabeculae that are resorbed in the remodeling process are preserved and even experience an increase in thickness resulting in a net zero chance in average thickness. This theory is supported by the current findings and also by the larger decrease in Tb.N. and only small change in thickness in long bones in similar OVX model characterization studies (12, 37, 40-42). OVX had a significant effect on DA, a morphological parameter, which describes the preferential alignment of the bone of the trabecular structure. An increasing value suggests preference for a more anisotropic structure, with a preferred orientation.
Our data are supportive of previous findings of an increase in DA following OVX in rat models of osteoporosis (43-45).

Over the 12-week study period, an interaction of hormone status and age of the rat was found for the cortical indices, BMD, Ps.Pm., and Ec.Pm. Interestingly, average Ps.Pm. and Ec.Pm. decreased over time, in such a way that Sham rats experienced the greatest decline in Ps.Pm. (3 %) and OVX rats the greatest decline in Ec.Pm. (54 %). It is generally understood that OVX causes bone loss on the endocortical surfaces (trabecular and endosteum) by causing an imbalance between bone formation and resorption (14, 17-19, 23). At first glance, our findings in OVX rats would imply that a decrease in Ec.Pm. was due to formation at this site. A more plausible mechanism could be that inner cortical bone perimeter may be decreasing with time because of a change in shape and, ultimately, an increased surface area. Bone at the endocortical surfaces may be resorbed into a more smooth and uniform shape, which is supported by the finding of an increase in Ct.Th. and Ct.Ar./Tt.Ar., and a decrease in Ma.Ar. and Ecc. over time. With the hormonal changes observed with age and with OVX, the marrow cavity expands and the cortical bone thins (17) in an effort to increase its resistance to bending by spreading its mass further away from the centroid, thereby increasing its moment of inertia. In this study, the cortical bone of the proximal tibia was analyzed. Although the cortical bone of the diaphysis of the rat experiences changes that are more consistent with the changes seen in the long bones of humans (46, 47), the diaphysis was not included in the single scan field, which was primarily aimed at the trabecular-rich proximal tibia region. To limit the length of time that the rat was anesthetized and the dose of radiation it was being exposed to, two scan fields were not practical in this study.
Of particular importance to note, trabecular BMD, BV/TV, Tb.Th., Tb.N., Tb.Sp., and cortical BMD of the proximal tibia were significantly different between treatment groups after only 1 week post-surgery. In this study, rats were ordered from the supplier either Sham-operated or ovariectomized at 12-weeks of age, and received their first scan after 1 week of recovery. Therefore, the earliest deterioration to bone microarchitecture was missed in our study. In the literature, the most rapid and detectable loss of bone was reported as early as 14 days post-OVX at the proximal tibia (23, 41) and more recent work using μCT at 26μm pixel size has found differences in trabecular outcomes 12 days post-OVX (36). Our findings that the trabecular bone microarchitecture and BMD show detectable differences between OVX and Sham rats after only 7 days post-surgery highlight the importance of timing in studies utilizing such sensitive, high-resolution imaging equipment. Efforts should be made to perform these surgeries on-site, and to acquire baseline values before any hormonal insult.

In conclusions, monthly irradiation over a 12-week period did not impact any of the microarchitectural properties of the proximal tibia, and this finding is consistent in both Sham and OVX rats. Radiation exposure affected only the 3D morphological parameter, cortical Ecc. Therefore, the rat tibia can be considered robust and resistant to radiation-induced damage under this scanning protocol. These findings can be used as proof of concept for the resilience of the rat tibia in intermittent in vivo radiation exposure of 600 mGy. This study also advances the characterization of the rat model of postmenopausal osteoporosis by providing a monthly time course of trabecular and cortical microarchitectural changes at the proximal tibia in rats. Future research should be done to elucidate any effect of repeated radiation exposure to the activity of bone
modeling/remodeling, as these processes may have been impacted without having had an
effect on microarchitectural properties as measured by μCT. Also, the use of this
scanning protocol should be reanalyzed using an experimental model of malignant bone
disease as this dose of radiation may have varying effects on different disease states.
References


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Chapter 6: Study 3

Comparison of *ex vivo* and *in vivo* μCT of Rat Tibia at Different Scanning Settings

Amanda B. Longo, Phil L. Salmon, and Wendy E. Ward

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Abstract

The parameters of a μCT scan, including whether a bone is imaged in vivo or ex vivo, determine the quality of the resulting image. In turn, this impacts the accuracy of the trabecular and cortical outcomes. The absolute impact of μCT scanning at different voxel sizes and whether the sample is imaged in vivo or ex vivo on the morphological outcomes of the proximal tibia in the rat is unknown. The right proximal tibia of 6-month old Sham-control and OVX rats (n=8/group) was scanned using μCT (SkyScan 1176, Bruker micro-CT, Kontich, Belgium) using three sets of parameters (9μm ex vivo, 18μm ex vivo, 18μm in vivo) to compare the trabecular and cortical outcomes. Regardless of scan protocols, differences between Sham and OVX groups were observed as expected. At a voxel size of 18μm, scanning in vivo or ex vivo had no effect on any of the outcomes measured. However, compared to a 9μm voxel size scan (ex vivo), imaging at 18μm ex vivo resulted in significant underestimation of the Conn.D (P<0.05) of the trabecular bone and a significant overestimation (P<0.05) of the trabecular indices (Tb.Th., DA) and of the cortical indices (Ct.Ar., Ct.Ar./Tt.Ar., Ct.Th.) in both Sham and OVX rats. These results suggest the benefit to scanning the proximal tibia of rats at a voxel size as low as 9μm, although considerations must be made for the increased acquisition time, anesthesia, animal welfare, and radiation exposure associated with lower voxel size in vivo scanning.

Introduction

The use of 3D μCT has become the gold standard for the quantitative assessment of bone microarchitecture (2). In vivo scanning allows for the repeated imaging and
analysis of the same animal over time in a longitudinal study. We have previously shown that repeated exposure of the rat hind limb to monthly radiation doses of 600 mGy does not cause differences in bone morphology, suggesting the resilience of the proximal tibia to repeated \textit{in vivo} \textmu CT scanning (3). Isolated bones however can be stored and scanned \textit{ex vivo} without consideration of scan duration, subsequent data file size, ionizing radiation, animal welfare, or artifacts caused by movement.

\textit{In vivo} imaging of the tibia involves scanning of the whole hind limb, including fur, skin, and soft tissue surrounding the bone, which may affect the image quality and subsequent outcomes. A greater volume of water surrounding an aluminum tube, used to represent bone, resulted in a reduction of beam hardening artifacts due to peripheral filtration of the X-rays away from the bone (4). By changing the scanning scenario from one material (aluminum) to two materials (aluminum surrounded by water), the need for beam hardening correction was reduced or eliminated (4, 5). Also, different thickness of surrounding medium can change the reconstructed attenuation so that the same bone in different parts of an animal can be assigned different density values, artificially. In general, the surrounding medium complicates the task of correcting beam hardening artifacts, which is necessary for the accurate quantification of bone densitometry and morphology. Furthermore, extra noise is added to reconstructed \textmu CT images by surrounding media.

In mice, scanning of the tibia \textit{ex vivo} with a 5\textmu m voxel size resulted in significant differences in some trabecular and cortical outcomes compared to \textit{in vivo} scanning with a 9\textmu m voxel size (6). The authors attribute these differences to the image quality and scanning resolution, but the effect of \textit{in vivo} and \textit{ex vivo} scanning cannot be discounted.
One study of female Wistar rats found all trabecular morphological outcomes of the tibia, as measured by µCT at a 26µm voxel size, were correlated between in vivo and ex vivo scanning protocols (7), but no study has directly compared these scan protocols at a smaller voxel size and in an OVX rat model, where a significant loss of trabecular bone volume and structure has occurred.

A 5µm voxel size is sufficient for the visualization and quantification of trabecular bone of the tibia of mice with only a minor benefit at higher resolutions (8). Trabeculae from ovary-intact rats are considerably thicker than mouse trabeculae, and scanning rat trabeculae at a 10-20µm voxel size is generally considered to be sufficient for imaging ex vivo (8), although no studies have directly compared the trabecular and cortical outcomes of the rat tibia scanned both in vivo and ex vivo at voxel sizes within this range. The OVX of 12-week SD rats causes an immediate and drastic deterioration in the morphology of the proximal tibia, including decreased trabecular bone volume, Tb.Th., Tb.N., and increased Tb.Sp. compared to their Sham-counterparts after only 7-days post-surgery (3). While the changes to bone as a result of OVX are drastic, it is important to determine whether these changes have an impact on the scan settings necessary for the accurate measurement and image quality. For this reason, both Sham- and OVX-operated rats were used to compare the right proximal tibia of rats scanned at voxel sizes of 9µm ex vivo, 18µm ex vivo and 18µm in vivo. The purpose of this study was to a) determine the effect of scanning the tibia of the rat in vivo compared to ex vivo b) further understand the implication of voxel size (9 versus 18µm) on the ex vivo scanning of the rat proximal tibia and to c) establish if the well characterized differences between Sham and OVX groups can be similarly detected with each scan protocol.
Findings from this study will guide the experimental design and determination of μCT scan parameters of future pre-clinical studies using the OVX rat model of postmenopausal osteoporosis.

**Materials and Methods**

*See Chapter Four – Experimental Approach for methodologies regarding Animals and Diet, μCT, and Statistical Analyses*

**Results**

Final body weights did not differ between Sham (390.8 ± 75.8 g) and OVX (422.2 ± 47.1 g) rats (P=0.237). Final food intake did not differ between Sham (14.7 ± 2.7 g/d) and OVX (14.0 ± 2.1 g/d) rats (P=0.607). Sham rats had significantly greater uterine wet weight at necropsy (660 ± 189.3 mg) compared to OVX rats (226.5 ± 26.7 mg) (P<0.001), confirming successful removal of the ovaries at 3 months of age.

In Sham rats, scanning of the right proximal tibia at a voxel size of 9μm ex vivo resulted in significantly lower values for the 3D trabecular morphometric indices Tb.Th. (P<0.001) and DA (P=0.001), and a greater Conn.D (P=0.009) and significantly lower values for the cortical parameter Ct.Ar. (P<0.001) compared to in vivo and ex vivo scanning protocols at the 18μm voxel size (Table 6-1). 9μm ex vivo scanning also resulted in lower Ct.Ar./Tt.Ar. (P=0.006) compared to 18μm in vivo scanning and lower Ct.Th. (P=0.007) compared to 18μm ex vivo scanning. No other trabecular or cortical morphometric outcomes were different among groups.

In OVX rats, scanning of the right proximal tibia at a voxel size of 9μm ex vivo resulted in significantly lower values for the trabecular morphometric indices Tb.Th. (P<0.001) and DA (P=0.002), and a greater Conn.D (P<0.001) and lower values for the
cortical indices Ct.Ar., Ct.Ar./Tt.Ar. (P<0.001) and Ct.Th. (P=0.001) compared to all other groups (Table 6-2). No other trabecular or cortical morphometric outcomes were different among groups.

R values for the correlations between 9µm and 18µm in vivo and 18µm ex vivo scan settings are reported in Table 6-3. Strong correlations were observed for most trabecular and cortical outcomes when Sham and OVX groups were analyzed together and many of these correlations were maintained when Sham and OVX groups were analyzed separately. Values for Tb.Th., DA, and Ct.Ar. when scanned at 18µm (in vivo or ex vivo) were not correlated with scans of 9µm.

Significant differences between Sham and OVX groups were observed for the outcomes BV/TV, Tb.N., Tb.Sp., Conn.D, Ct.Ar., Ct.Ar./Tt.Ar., Ct.Th., and Ec.Pm. when measured using all scanning protocols (Table 6-4). Scanning the proximal tibia at 18µm voxel size in vivo resulted in a significant difference between Sham and OVX rats for the trabecular outcome, Tb.Th. (P=0.012), but differences were not observed using other scanning protocols. Retrospective power analyses revealed the number of animals needed to identify differences between Sham and OVX groups for each of the primary outcome measures (Table 6-5).
Table 6-1. Trabecular and cortical outcomes of right proximal tibia of Sham-operated rats scanned using three sets of scanning parameters

<table>
<thead>
<tr>
<th>Scanning Parameters</th>
<th>BV/TV (%)</th>
<th>Tb.Th. (mm)</th>
<th>Tb.N. (mm⁻¹)</th>
<th>Tb.Sp. (mm)</th>
<th>Conn.D (mm⁻³)</th>
<th>DA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>9µm ex vivo</td>
<td>18µm ex vivo</td>
<td>18µm in vivo</td>
<td>ANOVA</td>
<td>9µm ex vivo</td>
<td>18µm ex vivo</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>18.2466 (6.6254)</td>
<td>22.2441 (7.9488)</td>
<td>24.8317 (3.0535)</td>
<td>0.128</td>
<td>(0.0067)ᵃ</td>
<td>(0.0065)ᵇ</td>
</tr>
<tr>
<td>Tb.Th. (mm)</td>
<td>0.0454 (0.0076)</td>
<td>0.0786 (0.0076)</td>
<td>0.0726 (0.0076)</td>
<td>0.552</td>
<td>(0.3578)²</td>
<td>(0.3926)²</td>
</tr>
<tr>
<td>Tb.N. (mm⁻¹)</td>
<td>4.0727 (1.7458)</td>
<td>2.7586 (1.0096)</td>
<td>3.4235 (0.3815)</td>
<td>0.110</td>
<td>(0.3578)²</td>
<td>(0.3926)²</td>
</tr>
<tr>
<td>Tb.Sp. (mm)</td>
<td>0.3594 (0.1767)</td>
<td>0.4116 (0.1767)</td>
<td>0.2449 (0.1767)</td>
<td>0.552</td>
<td>(0.3578)²</td>
<td>(0.3926)²</td>
</tr>
<tr>
<td>Conn.D (mm⁻³)</td>
<td>925.808 (822.021)</td>
<td>134.593 (84.078)</td>
<td>222.546 (66.257)</td>
<td>0.009</td>
<td>(0.3578)²</td>
<td>(0.3926)²</td>
</tr>
<tr>
<td>DA</td>
<td>1.5901 (0.1280)</td>
<td>1.9502 (0.2935)</td>
<td>1.9801 (0.0883)</td>
<td>0.001</td>
<td>(0.3578)²</td>
<td>(0.3926)²</td>
</tr>
</tbody>
</table>

Cortical Outcomes

<table>
<thead>
<tr>
<th></th>
<th>Tt.Ar. (mm²)</th>
<th>Ct.Ar. (mm²)</th>
<th>Ct.Ar./Tt.Ar. (%)</th>
<th>Ct.Th. (mm)</th>
<th>Ps.Pm. (mm)</th>
<th>Ec.Pm. (mm)</th>
<th>Ma.Ar. (mm²)</th>
<th>Ecc.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15.4514 (1.6288)</td>
<td>5.1653 (0.6401)ᵃ</td>
<td>33.797 (6.775)ᵃ</td>
<td>0.3923 (0.3032)ᵃ</td>
<td>33.0689 (3.1843)</td>
<td>1.6106 (0.4224)</td>
<td>30.2553 (4.9133)</td>
<td>0.3905 (0.0524)</td>
</tr>
<tr>
<td></td>
<td>15.7596 (1.6911)</td>
<td>6.3697 (0.3262)ᵇ</td>
<td>40.894 (5.505)ᵇ</td>
<td>0.4407 (0.0245)ᵇ</td>
<td>30.8204 (2.0532)</td>
<td>1.4303 (0.3872)</td>
<td>30.0481 (5.3022)</td>
<td>0.4172 (0.0723)</td>
</tr>
<tr>
<td></td>
<td>15.7149 (1.8830)</td>
<td>6.8426 (0.3986)ᵇ</td>
<td>43.934 (4.201)ᵇ</td>
<td>0.4233 (0.0280)ᵇ</td>
<td>30.7821 (2.1949)</td>
<td>1.6339 (0.2176)</td>
<td>30.8031 (5.8257)</td>
<td>0.4437 (0.0588)</td>
</tr>
<tr>
<td></td>
<td>0.929</td>
<td>&lt;0.001</td>
<td>0.006</td>
<td>0.007</td>
<td>0.142</td>
<td>0.465</td>
<td>0.959</td>
<td>0.249</td>
</tr>
</tbody>
</table>

Values are expressed as Mean (SD), n=8/group. Significant differences (P<0.05) are shown in bold type.ᵃᵇ Different letters across a row denote significant differences between all scanning parameters.
Table 6-2. Trabecular and cortical outcomes of right proximal tibia of OVX rats scanned using three sets of scanning parameters

<table>
<thead>
<tr>
<th>Trabecular Outcomes</th>
<th>Scanning Parameters</th>
<th>9µm ex vivo</th>
<th>18µm ex vivo</th>
<th>18µm in vivo</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV (%)</td>
<td></td>
<td>4.7031</td>
<td>5.7631</td>
<td>6.5859</td>
<td>0.165</td>
</tr>
<tr>
<td>(1.6105)</td>
<td></td>
<td>(2.0738)</td>
<td>(1.9987)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tb.Th. (mm)</td>
<td></td>
<td>0.0507</td>
<td>0.0848</td>
<td>0.0791</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(0.0039)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>(0.0072)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(0.0042)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tb.N. (mm&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td></td>
<td>0.9091</td>
<td>0.6521</td>
<td>0.8302</td>
<td>0.174</td>
</tr>
<tr>
<td>(0.3379)</td>
<td></td>
<td>(0.2232)</td>
<td>(0.2330)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tb.Sp. (mm)</td>
<td></td>
<td>1.1565</td>
<td>1.2536</td>
<td>1.1496</td>
<td>0.650</td>
</tr>
<tr>
<td>(0.2488)</td>
<td></td>
<td>(0.2397)</td>
<td>(0.2554)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conn.D (mm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td></td>
<td>145.445</td>
<td>17.867</td>
<td>46.880</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(45.819)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>(12.450)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(16.046)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DA</td>
<td></td>
<td>1.6450</td>
<td>2.1948</td>
<td>2.0159</td>
<td>0.002</td>
</tr>
<tr>
<td>(0.1747)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>(0.4091)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(0.1757)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cortical Outcomes</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tt.Ar. (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td></td>
<td>14.6701</td>
<td>15.0922</td>
<td>15.4852</td>
<td>0.292</td>
</tr>
<tr>
<td>(0.8249)</td>
<td></td>
<td>(1.0823)</td>
<td>(1.0950)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ct.Ar. (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td></td>
<td>6.0520</td>
<td>6.9295</td>
<td>7.4813</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(0.6379)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>(0.4164)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(0.4577)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ct.Ar./Tt.Ar. (%)</td>
<td></td>
<td>41.245</td>
<td>54.984</td>
<td>48.381</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(3.383)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>(1.992)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(2.022)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ct.Th. (mm)</td>
<td></td>
<td>0.4361</td>
<td>0.4775</td>
<td>0.4678</td>
<td>0.001</td>
</tr>
<tr>
<td>(0.0218)&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>(0.0146)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(0.0204)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ps.Pm. (mm)</td>
<td></td>
<td>31.7131</td>
<td>30.1985</td>
<td>30.8046</td>
<td>0.250</td>
</tr>
<tr>
<td>(2.4394)</td>
<td></td>
<td>(1.4249)</td>
<td>(1.1947)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ec.Pm. (mm)</td>
<td></td>
<td>1.1149</td>
<td>0.9199</td>
<td>1.1818</td>
<td>0.215</td>
</tr>
<tr>
<td>(0.4205)</td>
<td></td>
<td>(0.2206)</td>
<td>(0.2073)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ma.Ar. (mm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td></td>
<td>26.1494</td>
<td>26.3147</td>
<td>27.5346</td>
<td>0.514</td>
</tr>
<tr>
<td>(1.8549)</td>
<td></td>
<td>(3.0409)</td>
<td>(2.7024)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ecc.</td>
<td></td>
<td>0.4376</td>
<td>0.4438</td>
<td>0.4529</td>
<td>0.797</td>
</tr>
<tr>
<td>(0.0363)</td>
<td></td>
<td>(0.0595)</td>
<td>(0.0367)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as Mean (SD), n=8/group. Significant differences (P<0.05) are shown in bold type.<sup>a,b</sup> Different letters across a row denote significant differences between all scanning parameters.
Table 6-3. R values for the correlations between morphological outcomes from 18µm scanning protocols compared to 9µm *ex vivo* protocols

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>Sham</th>
<th>OVX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>18µm <em>ex vivo</em></td>
<td>18µm <em>in vivo</em></td>
<td>18µm <em>ex vivo</em></td>
</tr>
<tr>
<td><strong>Trabecular Outcomes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV</td>
<td>0.994**</td>
<td>0.913**</td>
<td>0.982*</td>
</tr>
<tr>
<td>Tb.Th.</td>
<td>-0.063</td>
<td>0.316</td>
<td>-0.145</td>
</tr>
<tr>
<td>Tb.N.</td>
<td>0.939**</td>
<td>0.860**</td>
<td>0.815*</td>
</tr>
<tr>
<td>Tb.Sp.</td>
<td>0.992**</td>
<td>0.967**</td>
<td>0.987**</td>
</tr>
<tr>
<td>Conn.D</td>
<td>0.401</td>
<td>0.567*</td>
<td>0.045</td>
</tr>
<tr>
<td>DA</td>
<td>0.114</td>
<td>0.396</td>
<td>-0.283</td>
</tr>
<tr>
<td><strong>Cortical Outcomes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tt.Ar.</td>
<td>0.950**</td>
<td>0.894**</td>
<td>0.918**</td>
</tr>
<tr>
<td>Ct.Ar.</td>
<td>0.446</td>
<td>0.184</td>
<td>0.298</td>
</tr>
<tr>
<td>Ct.Ar./Tt.Ar.</td>
<td>0.760**</td>
<td>0.696*</td>
<td>0.815*</td>
</tr>
<tr>
<td>Ct.Th.</td>
<td>0.767**</td>
<td>0.717*</td>
<td>0.765*</td>
</tr>
<tr>
<td>Ps.Pm.</td>
<td>0.846**</td>
<td>0.824**</td>
<td>0.847*</td>
</tr>
<tr>
<td>Ec.Pm.</td>
<td>0.770**</td>
<td>0.750**</td>
<td>0.690</td>
</tr>
<tr>
<td>Ma.Ar.</td>
<td>0.945**</td>
<td>0.954**</td>
<td>0.958**</td>
</tr>
<tr>
<td>Ecc</td>
<td>0.647*</td>
<td>0.430</td>
<td>0.562</td>
</tr>
</tbody>
</table>

The R values for the correlations between trabecular and cortical morphological outcomes from 18µm *ex vivo* and 18µm *in vivo* scan protocols compared to 9µm *ex vivo* scan protocols from all groups, Sham or OVX. Values with an * (P<0.05) or ** (P<0.001) are significantly correlated with outcomes obtained using 9µm *ex vivo* scan protocols.
Table 6-4. Statistical difference of trabecular and cortical outcomes between Sham and OVX groups for each scanning protocol

<table>
<thead>
<tr>
<th>Trabecular Outcomes</th>
<th>9µm ex vivo</th>
<th>18µm ex vivo</th>
<th>18µm in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tb.Th.</td>
<td>0.078</td>
<td>0.095</td>
<td>0.012</td>
</tr>
<tr>
<td>Tb.N.</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tb.Sp.</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Conn.D</td>
<td>0.035</td>
<td>0.011</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>DA</td>
<td>0.485</td>
<td>0.191</td>
<td>0.614</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cortical Outcomes</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tt.Ar.</td>
<td>0.253</td>
<td>0.363</td>
<td>0.771</td>
</tr>
<tr>
<td>Ct.Ar.</td>
<td>0.015</td>
<td>0.010</td>
<td>0.010</td>
</tr>
<tr>
<td>Ct.Ar./Tt.Ar.</td>
<td>0.017</td>
<td>0.028</td>
<td>0.022</td>
</tr>
<tr>
<td>Ct.Th.</td>
<td>0.005</td>
<td>0.003</td>
<td>0.003</td>
</tr>
<tr>
<td>Ps.Pm.</td>
<td>0.355</td>
<td>0.493</td>
<td>0.980</td>
</tr>
<tr>
<td>Ec.Pm.</td>
<td>0.034</td>
<td>0.006</td>
<td>0.001</td>
</tr>
<tr>
<td>Ma.Ar.</td>
<td>0.054</td>
<td>0.106</td>
<td>0.181</td>
</tr>
<tr>
<td>Ecc.</td>
<td>0.056</td>
<td>0.436</td>
<td>0.715</td>
</tr>
</tbody>
</table>

The p values of independent t-test between Sham and OVX groups are expressed, n=8/group. Significant differences (P<0.05) between Sham and OVX groups within each scanning protocol are shown in bold type.
Table 6-5. Sample size needed to identify differences in primary trabecular and cortical outcomes between Sham and OVX rats

<table>
<thead>
<tr>
<th>Trabecular Outcomes</th>
<th>Effect Size</th>
<th>9µm ex vivo</th>
<th>18µm ex vivo</th>
<th>18µm in vivo</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV</td>
<td>13.5433</td>
<td>7</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>Tb.Th.</td>
<td>0.0053</td>
<td>20</td>
<td>22</td>
<td>13</td>
</tr>
<tr>
<td>Tb.N.</td>
<td>3.1636</td>
<td>7</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Tb.Sp.</td>
<td>0.7971</td>
<td>7</td>
<td>7</td>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cortical Outcomes</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tt.Ar.</td>
<td>0.7813</td>
<td>45</td>
<td>71</td>
<td>622</td>
</tr>
<tr>
<td>Ct.Ar.</td>
<td>0.8867</td>
<td>12</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Ct.Ar./Tt.Ar.</td>
<td>7.273</td>
<td>13</td>
<td>14</td>
<td>13</td>
</tr>
<tr>
<td>Ma.Ar.</td>
<td>4.1059</td>
<td>17</td>
<td>24</td>
<td>33</td>
</tr>
</tbody>
</table>

Estimated number of rats necessary to identify differences between Sham and OVX groups for primary trabecular and cortical outcomes. Sample sizes were calculated with a power of 0.80, type I error rate of 0.05, and effect size and standard deviation from raw data.
Discussion

The purpose of this study was to better understand the implications of \textit{in vivo} and \textit{ex vivo} scanning as well as the effect of scan settings, including altered voxel size and rotation step, on trabecular and cortical outcomes in a pre-clinical model of postmenopausal osteoporosis. No differences were observed between \textit{in vivo} and \textit{ex vivo} scans of the proximal tibia at 18$\mu$m for any of the trabecular or cortical outcomes measured in Sham or OVX rats. This finding suggests that, despite the potential compromises to \textmu CT image quality imposed by \textit{in vivo} scanning, the quality of the resulting bone morphometric data is essentially equivalent to that of scans performed \textit{ex vivo} at the same voxel size. The reasoning for the lack of observed differences may be due to the immeasurable effect of the tissue surrounding the bone \textit{in vivo}. Ultimately, \textmu CT scanning at the proximal tibia whether performed \textit{in vivo} or free of all surrounding soft tissue does not result in changes to the trabecular or cortical outcomes assessed. In fact, a retrospective power analysis performed for each scan protocol revealed that when performing scans \textit{in vivo} a smaller sample size is necessary to identify differences in trabecular outcomes between Sham and OVX groups compared to \textit{ex vivo} techniques. Absolute resolution, the ability of a measure to quantify a theoretically perfect value, declines with increasing voxel size (9, 10). But, relative resolution, which is the ability of a scan to detect a difference between group A and B (i.e., Sham and OVX) can be unaffected with large increases in voxel size (11). Therefore, rather than the voxel size being the cause of the increase in effect size observed between Sham and OVX groups, there may be some unknown advantages of \textit{in vivo} imaging. While \textit{in vivo} scans are presumably subject to more artifacts, it is possible that \textit{in vivo} imaging provides a
reduction in the variability, as can be seen with a smaller standard deviation reported for most trabecular outcomes compared to *ex vivo* scans in this study. The surrounding tissue may act to hydrolyze the bone, maintain the bones size and shape, or reduce any potential post-mortem demineralization, suggesting a benefit to *in vivo* scanning of the hind limb in rats. The number of rats necessary to determine differences between Sham and OVX groups for the primary cortical outcomes were not affected by the scan parameter used. The effect sizes between Sham and OVX groups are larger for trabecular outcomes because of the rapid rate of bone loss on the trabecular surfaces compared to cortical (12). Therefore, when assessing changes to trabecular outcomes, fewer numbers of animals are needed.

In Sham and OVX rats, 18µm versus 9µm voxel size scanning overestimated Tb.Th., Ct.Ar., Ct.Th., and as a result, overestimated DA and Ct.Ar./Tt.Ar. and underestimated Conn.D. To calculate the morphological outcome Tb.Th. using the analysis program, the 3D image is first ‘skeletonized’ to identify the borders of solid trabecular structures and then ‘sphere-fitted’ to determine the average thickness of the largest voxel (3D pixel) that can fit within these borders. Tb.Sp. is quantified similarly, but the theory is applied to the space rather than the solid structures within the volume of interest (VOI). Figure 6-1 shows a comparison of the distribution of thickness of the trabeculae between scan protocols. Scanning at a lower voxel size causes a left shift in the distribution (thinner trabeculae), supporting the hypothesis that when scanning at a larger voxel size, thinner trabecular structures are not detected, leading to an artificial increase in the mean measured thickness. Also, with a larger voxel size, the surfaces of the bone tissue are blurred and the segmented surface becomes somewhat enlarged resulting in an
overestimation of thickness of the binarized surface. Ct.Th. is quantified in a similar way to Tb.Th.. Therefore scanning protocols with a greater voxel size will have the same overestimation effect as with the quantification of Tb.Th..
Figure 6-1. Distributions of the Tb.Th. of the proximal tibia between scan protocols in (A) Sham and (B) OVX rats

Solid, thick dash, and thin dash lines represent 9µm ex vivo, 18µm ex vivo, and 18µm in vivo scan protocols, respectively.
Along with percent bone volume, DA is an important determinant of mechanical strength of the trabecular bone (13). The DA of trabecular bone describes the orientation and alignment of the trabecular network within the VOI. The greater the preferential alignment of trabeculae along a principle axis the higher the DA. The alignment of the trabecular structure of the bone is measured in many orientations to determine the magnitude of the three principle vectors (Eigen values), from which DA is calculated. Scan acquisition with a larger voxel size and larger rotation step would limit the extent to which the trabecular network can be visualized and results in an overestimation of the DA, as seen in this study. Limited visualization of the trabecular network at larger voxel sizes also resulted in an underestimation of the Conn.D of the trabecular bone, a measure of the number of redundant connections between trabecular structures per unit volume. A larger Conn.D value implies a greater number of trabecular connections, which has been correlated with greater overall bone strength (14). Therefore, when comparing absolute values, scanning with greater voxel sizes can result in outcomes that are suggestive of a weaker bone due to a decreased Conn.D compared to scans done with smaller voxel sizes. Similar to the trabecular outcome DA, Ct.Ar. and Ct.Ar./Tt.Ar. are also overestimated with 18µm compared to 9µm voxel size scanning. Using 18µm, there is a smearing effect of the underlying structures (in this case, the edge of the cortical bone) and causes an overestimation of Ct.Th. because the boundaries are not as sharp and well defined. Part of this is due to increased instances of partial volume effect (15), which occurs when a single voxel on the border of a solid structure is made up of part bone and part empty space.
Overall, findings from this study report that scanning at different settings will result in different absolute morphological outcomes, but what may be of greater importance are the relative differences observed between scan settings. Most, but not all cortical and trabecular outcomes were correlated between 9µm and 18µm settings. The significant correlations that were observed between 9µm and 18µm scans suggest minimal relative changes between scan settings and support the use of 18µm voxel size scanning either in vivo or ex vivo when assessing relative changes at the proximal tibia between groups within a study. Depending on whether relationships were assessed between all 9µm and 18µm scans or within Sham and OVX groups independently, the correlations were similar. Taken together, this suggests that most bone morphological outcomes calculated from 9µm and 18µm voxel size scans are correlated regardless of whether they are used to assess groups with a high (Sham) or a low (OVX) bone mass.

The effect of OVX is robust, and as would be expected, caused significant and sustained differences in BV/TV, Tb.N., Tb.Sp., Conn.D, Ct.Ar., Ct.Ar./Tt.Ar., Ct.Th., and Ec.Pm. compared to Sham rats when assessed by all three scanning protocols at 6 months of age. A difference between OVX and Sham for the outcome Tb.Th. was observed only with 18µm in vivo scanning, suggesting that these different scan protocols alter the ability to differentiate between the absolute values of this trabecular outcome between Sham and OVX rats, only. Therefore, the use of different scanning protocols does not alter the ability to detect expected differences between Sham and OVX groups, suggesting that any unexpected differences observed are due to an intervention applied and not to the scanning protocols used.
While the benefits to in vivo µCT scanning are extensive, including the ability to follow a single subject longitudinally, there are also limits to this method. In vivo scanning requires the subject to be anesthetized for the duration of the scanning protocol which can become costly and depending on the frequency of the anesthesia, may affect the welfare of the animal. Very high-resolution in vivo imaging is also not the most optimal option when repeatedly scanning animals due to the extensive scan time and dose of ionizing radiation to the animal that is associated with these techniques. In this study, 9µm voxel size scans of the tibia required 44 minutes and created a 42GB file, compared to a scan time of 12 minutes and a file size of 7GB at a voxel size of 18µm. Although ex vivo scanning of isolated bones can only be analyzed at one time point, this method allows for less time-sensitive scanning protocols and for the use of smaller voxel sizes and rotation steps in order the acquire the highest quality image.

A specific limitation of this study was the timing between in vivo and ex vivo scanning. Rats were sacrificed within one-week following the in vivo scan, which may have allowed for a short period of remodeling and minor changes to bone structure. Although, an effect of growth cannot be fully discounted, only minor changes to the microarchitecture of the proximal tibia have been observed between 25 and 30 weeks post-OVX (16). A limitation of ex vivo scanning however, involves the inability to accurately and consistently measure BMD. After storage of isolated bones in saline-soaked gauze, large air-bubbles can be seen in the marrow space of the bone. These air-bubbles affect the calculation of Hounsfield units and ultimately, the determination of volumetric BMD. Finally, a true test of the effect of voxel size could not be conducted because 9µm settings used a smaller rotation step. Reducing the rotation step improves
the sharpness of the image and reduces noise, but all settings were chosen to provide a high quality image that can be used in future experimental settings.

In conclusion, the scan settings tested had no effect on the ability to observe expected differences between Sham and OVX groups highlighting the robust effect the loss of endogenous estrogen has on bone microarchitecture. *In vivo* scanning at 18µm voxel size in Sham or OVX rats at 6 months of age provides similar values to those obtained by *ex vivo* scanning at the same voxel size and most of these values are correlated with the values obtained using 9µm *ex vivo* scan settings. Together, these findings suggest no relative differences between findings from scan settings. However, scans performed using 18µm voxels, either *in vivo* or *ex vivo*, may overestimate or underestimate some morphological measurements at the proximal tibia compared to scans of 9µm voxels.
5. Meganck JA, Kozloff KM, Thornton MM, Broski SM, Goldstein SA. Beam hardening artifacts in micro-computed tomography scanning can be reduced by X-ray beam filtration and the resulting images can be used to accurately measure BMD. *Bone* 2009;45(6):1104-16
Chapter 7: Study 4

Lifelong Intake of Flaxseed oil or Menhaden oil to Provide Varying n-6 to n-3 PUFA Ratios Modulate Bone Microarchitecture During Growth, but Not After OVX in SD Rats
Abstract

Skeletal health is a lifelong process impacted by environmental factors, including nutrient intake. The n-3 source and PUFA ratio affect bone health in growing rats, or following OVX, but no study has investigated the longitudinal effect of PUFA-supplementation throughout these periods of bone development. One month old, SD rats (n=98) were randomized to receive 1 of 4 diets from 1 through 6 months of age. Diets were modified from AIN-93G to contain a varying amount and source of n-3 (flaxseed versus menhaden oil) to provide an n-6 to n-3 ratio of 10:1 or 5:1. At 3 (prior to SHAM or OVX) and 6 months of age, bone microarchitecture of the tibia was quantified using in vivo µCT (SkyScan 1176, Bruker microCT). Providing 5:1 (flaxseed) resulted in lower Tb.Th. and Ma.Ar. and greater Ct.Ar./Tt.Ar. during growth compared to diets with a 10:1 PUFA ratio, but many of these differences were not apparent following OVX. PUFA-supplementation at levels attainable in human diet modulates some bone structure outcomes during periods of growth, but is not an adequate strategy for the prevention of OVX-induced bone loss in rats.

Introduction

Coinciding with the transition to menopause, women experience a characteristic decrease in bone mass due to the decline in endogenous estrogen production, which ultimately increases the risk of fragility fracture and the development of osteoporosis [1]. The OVX rat model mimics the pathophysiology of bone loss in humans, making this an accepted model for the study of potential protective agents and strategies against the progression of osteoporosis [2-5].
The n-6 and n-3 series of PUFA have been shown to favourably modulate bone health in humans [6-12] and animal models [13-22]. Several feeding intervention trials in rats have been conducted to investigate either the source of n-3 PUFA, as ALA-rich or EPA and DHA-rich oils, or the ratio of n-6 to n-3 PUFA on bone health outcomes [13-22], but no study has directly compared these aspects. Moreover, previous studies of either the source or ratio of dietary PUFA can be categorized based on the life stage of the rat when the diet is consumed: during growth [13-15, 17] or following OVX [16, 18-22]. Studies have not examined the impact of varying the source or ratio of dietary PUFA in a longitudinal manner, spanning periods of development and aging. Therefore, this study investigated both the n-3 PUFA source and the ratio of n-6 to n-3 PUFA in rats from 1 through 6 months of age, to mimic the long-term exposure to these macronutrients consumed by humans.

In young rats undergoing periods of rapid bone growth, most work has been in male animal models and has investigated the effect of PUFA-supplementation on bone health outcomes including BMD, strength, calcium balance, and serum biomarkers of bone turnover [13-15, 23-27]. In general, these studies suggest a benefit to bone outcomes with a lower n-6 to n-3 PUFA ratio and differential effects depending on the predominant fatty acid found in each of the lipid sources tested. In females, feeding growing rats a diet containing menhaden oil for 5 weeks post-weaning had no effect on femoral BMD or biomechanical strength properties compared to control, although bone structure was not assessed [28]. Another study that did measure bone structure showed that feeding 28 day-old female rats flaxseed or menhaden oil compared to corn oil for 8 weeks resulted in a positive effect on trabecular microarchitecture of the femur and tibia.
This study investigated the effect of lipid source on bone structure with n-6 to n-3 PUFA ratios ranging from 1:3 through 48, unlike typical human dietary PUFA ratios which average 7 through 18:1 [29, 30]. No study has directly compared the effect of ALA-rich diets with diets rich in EPA+DHA in the OVX rat to determine their impact during periods predominated by bone remodeling.

Because n-6 and n-3 compete for the same pool of enzymes for their conversion to their longer chain derivatives and subsequent metabolites, the absolute amount of n-3 and resulting ratio of n-6 to n-3 PUFA is of particular importance. Studies have suggested a benefit to outcomes of bone health with the consumption of a lower n-6 to n-3 PUFA ratio in both growing [14, 17, 27] and OVX rats [16, 18, 19]. However, some studies supplement total PUFA levels that are supra-physiological and do not represent typical human consumption patterns [17, 18]. Therefore, the absolute amount of n-3 provided in the diets used in this study were modified to provide n-6 to n-3 PUFA at a 10:1 and 5:1 ratio, to test the effects of a dietary PUFA ratio above and below the average ratio consumed in Canada of approximately 7:1 [30]. These ratios were achieved by including either flaxseed or menhaden oil as the n-3 source. Diets provided a range of total n-6 PUFA and total n-3 PUFA between 12.8-14.8% kcal and 1.4-2.7% kcal, respectively (Table 4-1). The absolute amount of n-6 and n-3 PUFA provided in the intervention diets exceeded the average intakes in North American populations [30, 31]. In the USA, PUFA provide 8% total kcal with LA and ALA providing approximately 5.8 and 0.61% kcal, respectively [31]. In Canada, the average intake of total PUFA is 5.6% kcal, although the total LA or ALA intake has not been determined via nation-wide dietary recall survey [30]. Using these intervention diets, the objectives were to
determine the effect of varying i) the n-3 PUFA source (flaxseed versus menhaden oil), and ii) the absolute amount of n-3 provided to alter the n-6 to n-3 PUFA ratio (10:1 versus 5:1) on bone microarchitecture and volumetric BMD of the proximal tibia and lumbar vertebra 4 (L4) during periods of rapid growth and following Sham or OVX-surgery.

**Materials and Methods**

*See Chapter Four – Experimental Approach for methodologies regarding Animals and Diet, μCT, Serum Biomarker Analysis, and Statistical Analyses*

**Results**

*Uterine Weight, Body Weight, and Food Intake*

No significant interaction of diet and hormone status (p=0.354) or main effect of diet (p=0.301) was observed for uterine weights (data not shown). As expected, Sham rats had significantly greater (0.66 ± 0.14 g) uterine weight compared to OVX (0.18 ± 0.23 g) (p<0.001), confirming successful surgery at 3 months. Although near the level of significance, no interaction of diet and hormone status (p=0.054) or main effect of diet (p=0.544) was observed for body weight at any time throughout the study (Figure 7-1a). OVX rats had significantly greater body weight compared to Sham (p=0.006). Pairwise comparisons within time show that body weight at all time points was significantly different from one another (p<0.001) except for one week following surgery (p=0.074), suggesting continued growth throughout the study period except during recovery from surgery. No significant interaction (p=0.461), main effect of diet (p=0.666), or main effect of hormone status (p=0.079) was observed for food intake (Figure 7-1b).
Figure 7-1. (A) Weekly body weight and (B) three times weekly food intake from age 1 month through 6 months

The body weight of all rats was recorded once per week and the average food intake was recorded three times per week. Prior to Sham or OVX surgery (dotted line, open symbols), all rats were randomized to receive 1 of 4 intervention diets. At 3 months of age (solid line, closed symbols), rats were randomized to Sham or OVX surgery and were maintained on their respective diets until 6 months of age. Intervention diets and surgery-type are represented by symbol. All data are represented as mean (SEM).
In Vivo Microarchitecture of Tibia

At 3 months of age, rats receiving 5:1 (flaxseed) diets had significantly lower Tb.Th. and Ma.Ar. compared to rats receiving 10:1 (flaxseed) and greater Ct.Ar./Tt.Ar. of the tibia compared to rats receiving either 10:1 diet (Table 7-1). No significant interaction of diet and hormone status or main effect of diet was observed for any trabecular outcome of the tibia at 6 months of age (data not shown). A main effect of diet was observed for the cortical indices BMD, Ct.Ar./Tt.Ar. and Ma.Ar. at 6 months of age (p<0.05) (data not shown). Post hoc analysis revealed 10:1 (flaxseed) and 5:1 (flaxseed) fed groups experienced lower cortical BMD and Ma.Ar. compared to all other groups, respectively (p<0.05). 5:1 (flaxseed) fed groups experienced greater Ct.Ar./Tt.Ar. compared to both 10:1 fed groups. Hormone status significantly affected Ct.Ar./Tt.Ar. (p<0.05), trabecular BMD, BV/TV, Tb.N., Tb.Sp. and Ec.Pm. (p<0.001) at 6 months of age (data not shown).

When the absolute change from 3 to 6 months was examined, no significant interaction of diet and hormone status was observed. Diet had a main effect on cortical BMD (p<0.05) with 10:1 (flaxseed) fed groups experiencing the greatest loss compared to other groups (data not shown). Hormone status significantly affected Ct.Ar./Tt.Ar., Ec.Pm., Ma.Ar. (p<0.05), trabecular BMD, BV/TV, Tb.N., Tb.Sp. and Ps.Pm. (p<0.001).

Sham rats receiving 5:1 (flaxseed) diets experienced lower trabecular BMD, BV/TV, Tb.N., and greater Tb.Sp. of the tibia compared to Sham rats receiving 5:1 (menhaden) diets (Table 7-2a). These diet-induced effects on trabecular outcomes of the tibia were not observed in OVX rats (Table 7-2b). In rats randomized to OVX surgery, those fed diets of 5:1 (flaxseed) experienced lower Ma.Ar. compared to 10:1 (flaxseed)
fed rats. Feeding 5:1 (menhaden) resulted in greater Ct.Th. compared to all other OVX groups. Feeding diets with a 10:1 n-6 to n-3 PUFA ratio resulted in greater Ct.Ar./Tt.Ar. compared to 5:1 fed rats. These effects of diet to the cortical outcomes of the tibia were not observed in Sham rats.
### Table 7-1. *In vivo* microarchitecture of proximal tibia from 3 month old rats

<table>
<thead>
<tr>
<th></th>
<th>10:1 Flaxseed n=25</th>
<th>5:1 Flaxseed n=25</th>
<th>10:1 Menhaden n=24</th>
<th>5:1 Menhaden n=24</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trabecular Outcomes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD (g/cm(^3))</td>
<td>0.243 (.083)</td>
<td>0.217 (.095)</td>
<td>0.230 (.091)</td>
<td>0.260 (.083)</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>20.53 (4.37)</td>
<td>18.95 (5.01)</td>
<td>19.44 (4.57)</td>
<td>21.23 (4.24)</td>
</tr>
<tr>
<td>Tb.Th. (mm)</td>
<td>0.078 (.004)</td>
<td>0.075 (.004)</td>
<td>0.077 (.004)</td>
<td>0.076 (.005)</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>a, b</td>
<td></td>
</tr>
<tr>
<td>Tb.N. (mm(^3))</td>
<td>2.639 (1.495)</td>
<td>2.540 (1.706)</td>
<td>2.549 (1.651)</td>
<td>2.800 (1.528)</td>
</tr>
<tr>
<td>Tb.Sp. (mm)</td>
<td>0.360 (.132)</td>
<td>0.442 (.207)</td>
<td>0.423 (.177)</td>
<td>0.370 (.158)</td>
</tr>
<tr>
<td><strong>Cortical Outcomes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD (g/cm(^3))</td>
<td>1.800 (.006)</td>
<td>1.795 (.015)</td>
<td>1.799 (.008)</td>
<td>1.798 (.013)</td>
</tr>
<tr>
<td>Ct.Ar./Tt.Ar. (%)</td>
<td>37.75 (3.65)</td>
<td>40.09 (4.47)</td>
<td>37.93 (3.16)</td>
<td>38.73 (2.99)</td>
</tr>
<tr>
<td>Ct.Th. (mm)</td>
<td>0.355 (.026)</td>
<td>0.354 (.030)</td>
<td>0.352 (.027)</td>
<td>0.362 (.024)</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>a, b</td>
<td></td>
</tr>
<tr>
<td>Ps.Pm. (mm)</td>
<td>31.250 (1.796)</td>
<td>31.024 (2.638)</td>
<td>31.327 (1.812)</td>
<td>30.759 (1.849)</td>
</tr>
<tr>
<td>Ec.Pm. (mm)</td>
<td>1.606 (.344)</td>
<td>1.645 (.839)</td>
<td>1.579 (.287)</td>
<td>1.593 (.372)</td>
</tr>
<tr>
<td>Ma.Ar. (mm(^2))</td>
<td>33.821 (5.925)</td>
<td>30.413 (6.213)</td>
<td>33.253 (5.263)</td>
<td>32.482 (5.118)</td>
</tr>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>a, b</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as Mean (SD), n=24-25/group. Different letters in a row denote significant differences between diet groups. Significance was set at p<0.05.

BMD, bone mineral density; BV/TV, percent bone volume; Ct.Ar./Tt.Ar., cortical area fraction; Ct.Th., cortical thickness; Ec.Pm., endocortical perimeter; Ma.Ar., medullary area; Ps.Pm., periosteal perimeter; Tb.N., trabecular number; Tb.Sp., trabecular separation; Tb.Th., trabecular thickness.
Table 7-2. *In vivo* microarchitecture of the proximal tibia from 6 month old (A) Sham and (B) OVX rats

(A) Sham

<table>
<thead>
<tr>
<th></th>
<th>10:1 Flaxseed n=12</th>
<th>5:1 Flaxseed n=12</th>
<th>10:1 Menhaden n=12</th>
<th>5:1 Menhaden n=12</th>
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</thead>
<tbody>
<tr>
<td><strong>Trabecular Outcomes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD (g/cm³)</td>
<td>0.377</td>
<td>0.334</td>
<td>0.353</td>
<td>0.413</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>23.91</td>
<td>21.00</td>
<td>22.26</td>
<td>26.29</td>
</tr>
<tr>
<td>Tb.Th. (mm)</td>
<td>0.077</td>
<td>0.076</td>
<td>0.074</td>
<td>0.073</td>
</tr>
<tr>
<td>Tb.N. (mm⁻¹)</td>
<td>3.100</td>
<td>2.739</td>
<td>3.020</td>
<td>3.614</td>
</tr>
<tr>
<td><strong>Cortical Outcomes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD (g/cm³)</td>
<td>1.634</td>
<td>1.750</td>
<td>1.750</td>
<td>1.752</td>
</tr>
<tr>
<td>Ct.Ar./Tt.Ar. (%)</td>
<td>44.64</td>
<td>46.41</td>
<td>44.61</td>
<td>43.95</td>
</tr>
<tr>
<td>Ct.Th. (mm)</td>
<td>0.430</td>
<td>0.436</td>
<td>0.433</td>
<td>0.431</td>
</tr>
<tr>
<td>Ps.Pm. (mm)</td>
<td>30.261</td>
<td>29.750</td>
<td>30.686</td>
<td>31.202</td>
</tr>
<tr>
<td>Ec.Pm. (mm)</td>
<td>1.543</td>
<td>1.574</td>
<td>1.854</td>
<td>1.888</td>
</tr>
<tr>
<td>Ma.Ar. (mm²)</td>
<td>30.236</td>
<td>27.668</td>
<td>30.320</td>
<td>32.035</td>
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</table>
(B) OVX

<table>
<thead>
<tr>
<th>Trabecular Outcomes</th>
<th>10:1 Flaxseed &amp; 5:1 Flaxseed</th>
<th>10:1 Menhaden &amp; 5:1 Menhaden</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>n=13</td>
<td>n=13</td>
</tr>
<tr>
<td>BMD (g/cm$^3$)</td>
<td>0.128 (0.050)</td>
<td>0.117 (0.050)</td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>7.05 (3.30)</td>
<td>6.35 (3.34)</td>
</tr>
<tr>
<td>Tb.Th. (mm)</td>
<td>0.076 (0.009)</td>
<td>0.075 (0.007)</td>
</tr>
<tr>
<td>Tb.N. (mm$^3$)</td>
<td>0.958 (.490)</td>
<td>0.868 (.528)</td>
</tr>
<tr>
<td>Tb.Sp. (mm)</td>
<td>1.150 (.365)</td>
<td>1.180 (.398)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cortical Outcomes</th>
<th>10:1 Flaxseed &amp; 5:1 Flaxseed</th>
<th>10:1 Menhaden &amp; 5:1 Menhaden</th>
</tr>
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<tr>
<td></td>
<td>n=13</td>
<td>n=13</td>
</tr>
<tr>
<td>BMD (g/cm$^3$)</td>
<td>1.608 (.180)</td>
<td>1.688 (.167)</td>
</tr>
<tr>
<td>Ct.Ar./Tt.Ar. (%)</td>
<td>44.63 (3.43)$^a$</td>
<td>48.68 (3.77)$^b$</td>
</tr>
<tr>
<td>Ct.Th. (mm)</td>
<td>0.435 (.025)$^a$</td>
<td>0.432 (.029)$^a$</td>
</tr>
<tr>
<td>Ps.Pm. (mm)</td>
<td>31.613 (1.421)</td>
<td>31.249 (1.918)</td>
</tr>
<tr>
<td>Ec.Pm. (mm)</td>
<td>1.375 (.701)</td>
<td>1.297 (.473)</td>
</tr>
<tr>
<td>Ma.Ar. (mm$^2$)</td>
<td>30.915 (4.442)$^a$</td>
<td>25.652 (4.124)$^b$</td>
</tr>
</tbody>
</table>

Values are expressed as Mean (SD), n=12-13/group. Different letters in a row denote significant differences between diet groups and hormone status. Significance was set at p<0.05.

BMD, bone mineral density; BV/TV, percent bone volume; Ct.Ar./Tt.Ar., cortical area fraction; Ct.Th., cortical thickness; Ec.Pm., endocortical perimeter; Ma.Ar., medullary area; Ps.Pm., periosteal perimeter; Tb.N., trabecular number; Tb.Sp., trabecular separation; Tb.Th., trabecular thickness.
Ex vivo Microarchitecture of LV4

At 3 months of age, rats receiving diets of 10:1 (Menhaden oil) experienced reduced DA compared to rats receiving diets with n-3 source from flaxseed (Table 7-3). At 6 months of age, there was no interaction of diet and hormone status observed for the trabecular outcomes of LV4. Diet had a main effect on BV/TV and Tb.N. \((p<0.05)\), and as expected, hormone status had a main effect on all trabecular outcomes, except Tb.Th. \((p<0.001)\) (Table 7-4). Sham rats randomized to receive diets of 5:1 (menhaden) had a greater Tb.N. compared to Sham rats randomized to diets of 10:1 (flaxseed) (Table 7-4a). Diet had no significant effect on trabecular outcomes of LV4 in rats randomized to OVX (Table 7-4b).
Table 7-3. *Ex vivo* microarchitecture of LV4 from 3 month old rats.

<table>
<thead>
<tr>
<th>Trabecular Outcomes</th>
<th>10:1 (Flaxseed oil)</th>
<th>5:1 (Flaxseed oil)</th>
<th>10:1 (Menhaden oil)</th>
<th>5:1 (Menhaden oil)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV (%)</td>
<td>20.271 (3.155)</td>
<td>20.202 (2.420)</td>
<td>24.301 (5.355)</td>
<td>26.050 (3.344)</td>
</tr>
<tr>
<td>Tb.Th. (mm)</td>
<td>0.076</td>
<td>0.076</td>
<td>0.078</td>
<td>0.079</td>
</tr>
<tr>
<td>Tb.N. (mm&lt;sup&gt;1&lt;/sup&gt;)</td>
<td>2.658 (0.004)</td>
<td>2.668 (0.003)</td>
<td>3.094</td>
<td>3.316</td>
</tr>
<tr>
<td>Tb.Sp. (mm)</td>
<td>0.295</td>
<td>0.289</td>
<td>0.263</td>
<td>0.248</td>
</tr>
<tr>
<td>DA</td>
<td>2.093</td>
<td>2.086</td>
<td>1.886</td>
<td>1.905</td>
</tr>
<tr>
<td>Conn.D (mm&lt;sup&gt;3&lt;/sup&gt;)</td>
<td>85.249 (9.297)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>95.702 (11.774)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>87.181 (9.281)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>90.262 (10.357)&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are expressed as Mean (SD), n=4-6/group. Different letters in a row denote significant differences between diet groups. Significance was set at p<0.05.

BV/TV, percent bone volume; Conn.D; connectivity density, DA; degree of anisotropy, Tb.N.; trabecular number, Tb.Sp.; trabecular separation, Tb.Th.; trabecular thickness.
Table 7-4. *Ex vivo* microarchitecture of LV4 from 6 month old (A) Sham and (B) OVX rats.

(A) Sham

<table>
<thead>
<tr>
<th></th>
<th>10:1 Flaxseed n=13</th>
<th>5:1 Flaxseed n=13</th>
<th>10:1 Menhaden n=12</th>
<th>5:1 Menhaden n=12</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trabecular Outcomes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>25.609 (4.383)</td>
<td>22.634 (2.592)</td>
<td>23.884 (3.154)</td>
<td>26.331 (4.295)</td>
</tr>
<tr>
<td>Tb.Th. (mm)</td>
<td>0.081 (.006)</td>
<td>0.082 (.005)</td>
<td>0.081 (.005)</td>
<td>0.083 (.005)</td>
</tr>
<tr>
<td>Tb.N. (mm$^{-1}$)</td>
<td>3.162 (.448)$^{ab}$</td>
<td>2.765 (.252)$^a$</td>
<td>2.932 (.329)$^{ab}$</td>
<td>3.151 (.375)$^b$</td>
</tr>
<tr>
<td>Tb.Sp. (mm)</td>
<td>0.259 (.028)</td>
<td>0.293 (.025)</td>
<td>0.276 (.024)</td>
<td>0.260 (.201)</td>
</tr>
</tbody>
</table>

(B) OVX

<table>
<thead>
<tr>
<th></th>
<th>10:1 Flaxseed n=13</th>
<th>5:1 Flaxseed n=13</th>
<th>10:1 Menhaden n=12</th>
<th>5:1 Menhaden n=12</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trabecular Outcomes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BV/TV (%)</td>
<td>15.845 (4.204)</td>
<td>14.293 (3.762)</td>
<td>16.925 (2.851)</td>
<td>16.297 (3.149)</td>
</tr>
<tr>
<td>Tb.Th. (mm)</td>
<td>0.083 (.007)</td>
<td>0.083 (.007)</td>
<td>0.084 (.006)</td>
<td>0.082 (.004)</td>
</tr>
<tr>
<td>Tb.N. (mm$^{-1}$)</td>
<td>1.900 (.439)</td>
<td>1.736 (.489)</td>
<td>2.008 (.291)</td>
<td>1.985 (.317)</td>
</tr>
<tr>
<td>Tb.Sp. (mm)</td>
<td>0.393 (.062)</td>
<td>0.421 (.086)</td>
<td>0.384 (.060)</td>
<td>0.383 (.061)</td>
</tr>
</tbody>
</table>

Values are expressed as Mean (SD), n=12-13/group. Different letters in a row denote significant differences between diet groups and hormone status. Significance was set at p<0.05.

BV/TV, percent bone volume; Tb.N., trabecular number; Tb.Sp., trabecular separation; Tb.Th., trabecular thickness.
Serum OPG and RANKL and the ratio of OPG to RANKL

No interaction or main effect of diet was observed for the serum biomarkers OPG (Interaction, p=0.243; Diet, p=0.331), RANKL (Interaction, p=0.912; Diet, p=0.922), or the ratio of OPG to RANKL (Interaction, p=0.429; Diet, p=0.452) (Figure 7-2). OVX resulted in lower OPG concentrations and a lower ratio of OPG to RANKL and higher RANKL concentrations compared to Sham (p<0.001).
Figure 7-2. Serum (A) OPG and (B) RANKL concentrations and the (C) ratio of OPG to RANKL from 6 month old rats

Values are expressed as Mean (SD). Light shaded bars represent Sham. Dark shaded bars represent OVX. *Significantly different from Sham (p<0.05).
Body Composition

A significant interaction of hormone status and diet was observed for the percent lean mass volume, VOI length, and lean volume relative to VOI length (p<0.05) (Table 7-5). No main effect of hormone status was observed, but diet had a significant main effect on percent lean mass volume and lean volume relative to VOI length (p<0.001). Post hoc analysis revealed Sham rats receiving 5:1 (Menhaden oil) diets had significantly lower percent lean mass volume compared to all other Sham rats and greater non-lean volume relative to VOI length compared to 5:1 (Flaxseed) fed rats (Table 7-5).
Table 7-5. *In vivo* body composition of abdominal region surrounding LV3 from 6 month old rats

<table>
<thead>
<tr>
<th></th>
<th>10:1 (Flaxseed oil)</th>
<th>5:1 (Flaxseed oil)</th>
<th>10:1 (Menhaden oil)</th>
<th>5:1 (Menhaden oil)</th>
<th>10:1 (Flaxseed oil)</th>
<th>5:1 (Flaxseed oil)</th>
<th>10:1 (Menhaden oil)</th>
<th>5:1 (Menhaden oil)</th>
<th>6 months</th>
<th>X</th>
<th>Diet</th>
<th>HS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Composition Outcomes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent Non-Lean Volume</td>
<td>17.83 (5.56)</td>
<td>17.12 (4.68)</td>
<td>18.33 (4.35)</td>
<td>19.50 (5.26)</td>
<td>19.36 (5.20)</td>
<td>18.71 (8.06)</td>
<td>21.01 (7.15)</td>
<td>19.95 (5.38)</td>
<td>0.910</td>
<td>0.749</td>
<td>0.212</td>
<td></td>
</tr>
<tr>
<td>Percent Lean Mass Volume</td>
<td>59.47 (8.53)*</td>
<td>59.15 (7.97)*</td>
<td>53.17 (10.97)a</td>
<td>39.24 (12.56)b</td>
<td>56.56 (8.90)</td>
<td>57.16 (11.21)</td>
<td>46.68 (16.42)</td>
<td>51.62 (10.36)*</td>
<td><strong>0.035</strong></td>
<td>&lt;0.001</td>
<td>0.979</td>
<td></td>
</tr>
<tr>
<td>Percent Bone Volume</td>
<td>1.46 (.31)</td>
<td>1.38 (.29)</td>
<td>1.38 (.26)</td>
<td>1.44 (.30)</td>
<td>1.22 (.32)</td>
<td>2.03 (2.50)</td>
<td>1.47 (.20)</td>
<td>1.173 (.31)</td>
<td>0.324</td>
<td>0.470</td>
<td>0.711</td>
<td></td>
</tr>
<tr>
<td>VOI Length (mm)</td>
<td>8.408 (.342)</td>
<td>8.025 (.352)</td>
<td>8.351 (.320)</td>
<td>8.487 (.580)</td>
<td>8.335 (.266)</td>
<td>8.335 (.412)*</td>
<td>8.234 (.490)</td>
<td>8.204 (.244)</td>
<td><strong>0.045</strong></td>
<td>0.404</td>
<td>0.646</td>
<td></td>
</tr>
<tr>
<td>Non-Lean Volume/VOI Length</td>
<td>2.11 (.64)</td>
<td>2.13 (.56)</td>
<td>2.19 (.49)</td>
<td>2.29 (.57)</td>
<td>2.32 (.62)</td>
<td>2.23 (.91)</td>
<td>2.55 (.86)</td>
<td>2.42 (.62)*</td>
<td>0.924</td>
<td>0.771</td>
<td>0.169</td>
<td></td>
</tr>
<tr>
<td>Lean Volume/VOI Length</td>
<td>7.10 (1.15)</td>
<td>7.40 (1.13)</td>
<td>6.36 (1.29)</td>
<td>4.59 (1.33)</td>
<td>6.80 (1.15)</td>
<td>6.90 (1.51)</td>
<td>5.66 (2.01)</td>
<td>6.30 (1.32)</td>
<td><strong>0.018</strong></td>
<td>&lt;0.001</td>
<td>0.597</td>
<td></td>
</tr>
<tr>
<td>Bone Volume/VOI Length</td>
<td>0.17 (.04)</td>
<td>0.17 (.04)</td>
<td>0.17 (.03)</td>
<td>0.17 (.04)</td>
<td>0.15 (.04)</td>
<td>0.24 (.03)</td>
<td>0.16 (.06)</td>
<td>0.14 (.04)</td>
<td>0.401</td>
<td>0.404</td>
<td>0.767</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as Mean (SD), n=10-13/group. Different letters in a row denote significant differences between diet groups and hormone status (HS). * Significantly different from corresponding SHAM value. Significance was set at p<0.05. Non-lean volume excludes all lean and bone tissue.
Discussion

This study was the first to investigate the effect of feeding a varying PUFA ratio, attained by providing two levels of n-3 PUFA, at physiologically relevant levels, and to directly compare the n-3 PUFA sources (ALA-rich flaxseed oil versus EPA+DHA-rich menhaden oil) over the life course. Consistent changes to the primary microarchitectural outcomes of trabecular (BV/TV) and cortical (Ct.Ar./Tt.Ar. and Ct.Th.) bone were largely not observed among groups. These primary outcomes of bone structure are most closely related to alterations in functional outcomes, including bone strength and ultimately to resistance to fracture [34, 35]. Some secondary outcomes, that are unlikely to affect bone strength independent of changes to primary outcomes, were altered but many of these differences are difficult to interpret or extrapolate without concomitant changes to primary outcomes. However, it is still important to measure the effect of an intervention to secondary outcomes to understand the time-course of bone structure changes leading to differences in primary outcomes. Although limited consistency in changes to BMD, BV/TV, Ct.Ar./Tt.Ar. and Ct.Th. were observed in this study, differences due to diet may have been found with a longer follow-up period post-surgery.

Overall, rats experienced small differences, mainly in secondary outcomes, to microarchitecture of the tibia following periods of rapid growth (up to 3 months of age) and the lower Ma.Ar. and higher Ct.Ar./Tt.Ar observed with 5:1 (flaxseed) feeding compared to 10:1 fed groups were sustained to 6 months of age. At 6 months of age, diet influenced cortical BMD, Ct.Ar./Tt.Ar. and Ma.Ar., with the greatest detriments to bone health observed in 10:1 (flaxseed) fed groups. However, when the absolute changes between 3 and 6 months of age were assessed, only the detriments of 10:1 (flaxseed)
feeding to cortical BMD persisted, suggesting that varying PUFA ratio and n-3 source is not an adequate strategy to protect against OVX-induced bone loss.

Following a period of rapid bone growth, feeding diets with a lower ratio of n-6 to n-3 PUFA with n-3 provided from flaxseed oil resulted in a lower Tb.Th., suggesting an overall thinning of the trabecular structures of the tibia. As there were no differences between diet groups for the other trabecular morphology related structures, this finding is generally considered to be detrimental to overall bone health. Flaxseed (5:1) fed rats also experienced higher Ct.Ar./Tt.Ar., implying a benefit to the cortical structure, and a lower Ma.Ar. compared to diets with a higher PUFA ratio, which persisted to 6 months of age. Without concomitant changes to Ps.Pm. or Ec.Pm., it is difficult to interpret how a lower Ma.Ar. impacts bone health in this rat model. At 6 months of age, there was no effect of diet on the trabecular bone, however the diet-induced effects of 5:1 (flaxseed) of the cortical bone at 3 months of age were sustained to 6 months of age, suggesting that the greater volume fraction of cortical bone acquired during periods of rapid growth are maintained following Sham or OVX surgery. The changes to cortical bone microarchitecture with diet were not reflected in serum biomarkers of bone formation (OPG) or resorption (RANKL) or their ratio at study endpoint. OVX- and diet-induced effects on markers of bone turnover occur rapidly in rat blood and skeletal tissue [36, 37] and so the lack of differences observed may be due to the extended length of time of the intervention period, which spanned 5 months.

Because of the longitudinal design of this study, the absolute change in outcomes from 3 through 6 months of age was analyzed. This secondary analysis allowed us to isolate the effect of the intervention diets following surgery without the confounding
effect of the diet fed during rapid bone growth (up to 3 months of age) and showed that none of the differences observed at 3 months were sustained. This suggests the proximal tibia is sensitive to dietary influence during periods of rapid growth, and some of the effects are maintained to 6 months of age, but the bone is no longer as responsive to diet after having undergone Sham or OVX surgery. In fact, the only outcome to be affected by diet from 3 to 6 months of age was cortical BMD, with 10:1 (flaxseed) fed rats experiencing the greatest decline following surgery.

As expected, OVX caused significant detriments to trabecular bone compared to Sham rats [5]. For this reason, Sham and OVX rats were analyzed independently so that subtle effects of diet were not overshadowed by the dramatic influence of hormone status. Similar to previous research, diet was shown to have effects specific to hormone status, suggesting that bone from Sham and OVX rats does not respond to PUFA-feeding trials similarly [22]. Feeding 5:1 (menhaden) resulted in consistent benefits to most trabecular bone outcomes (compared to 5:1 (flaxseed)) and to Ct.Th, (compared to all diets) in Sham and OVX rats, respectively. This suggests some effect of PUFA supplementation in circumstances either with or without endogenous hormone production, but not the same effects under both conditions. A possible reason for the opposing diet-induced changes to bone microarchitecture may involve the amount of bone tissue that can be modulated. With aging and following OVX, trabecular bone loss proceeds more rapidly than cortical bone loss simply because of the accessibility of bone surface area for remodeling to initiate [38, 39]. In OVX rats that experience rapid trabecular bone loss, PUFA-supplementation may only have the opportunity to influence cortical bone as most trabeculae are resorbed within 1 month following surgery [5]. Therefore, feeding a diet
with PUFA levels that are attainable through a healthful diet alone, without supplementation, may not be adequate to protect against the rapid and substantial deterioration of trabecular bone tissue at the proximal tibia or lumbar vertebrae following OVX at 3 months of age in the rat.

Contrary to our hypothesis, no consistent benefits of either n-3 source were observed at either time point. In general, a benefit to trabecular bone was found with diets supplemented with menhaden oil compared to flaxseed oil, but no consistent effect of either n-3 source was observed for cortical bone outcomes. ALA is the predominant n-3 PUFA found in flaxseed oil and can be converted to EPA and DHA, although this process is relatively inefficient [40]. Therefore, results from this study suggest that dietary ALA does not produce an equivalent biological response to EPA+DHA-rich lipid sources. In a previous study of young female rats, feeding either flaxseed or menhaden oil caused significant increases in femur BV/TV compared to corn oil control, but no differences were observed between these two n-3 sources [17].

In general, a benefit to bone health was observed with a lower n-6 to n-3 PUFA ratio, although these observed benefits were not consistent. Previous research in growing and OVX rats has suggested benefits of a lower PUFA ratio [14, 16-19, 27]; these ratios often do not represent normal dietary intake patterns. Some controversy surrounding the relevancy of the importance of the n-6 to n-3 PUFA ratio has arisen in relation to cardiovascular disease risk [41], although limited studies have been aimed at assessing bone health outcomes. Rather, the absolute amount of n-3 PUFA in the diet may be of more importance [42, 43]. Although the current study found no consistent implications to bone health with varying the absolute amounts of n-3 PUFA, more research should be
undertaken to further elucidate the influence of n-6 and n-3 PUFA ratio in the diet and its effect on bone microarchitecture changes. Therefore, the consumption of a low n-6 to n-3 PUFA ratio with a balance of both ALA and EPA+DHA-rich sources may be the most appropriate strategy for the attainment and preservation of bone mass and microarchitecture, rather than focusing on a single fatty acid source.

In contrast to many findings reported in the literature, hormone status did not have an effect on lean, non-lean, or bone mass. Following OVX, rats experience significant increases in fat mass compared to their Sham counterparts [44]. This discrepancy may be due to the relatively small ROI under investigation. Body composition was measured in the abdominal region surrounding LV3 only, a much smaller region than would be measured using DXA. Diet did however have an effect on body composition, whereby within Sham rats, those receiving diets of 5:1 (menhaden) had lower percent lean mass volume compared to all other Sham rats and greater non-lean volume relative to VOI length compared to 5:1 (flaxseed) fed Sham rats. Few studies in the literature have directly compared the source of n-3 on body composition of the rat, but supplementation of male Wistar rats with EPA has resulted in a decreased total body lean mass compared to control feeding [45] when ALA-supplementation did not. The reduction in non-lean volume with flaxseed supplementation observed in this study has been supported in studies of young male Wistar rats [46], male rats post-weaning [47], and during lactation [48]. Future research should be undertaken to determine the varying effects of n-3 source and PUFA ratio on the changes to body composition within a larger VOI throughout the lifespan in the rat.
The experimental design of this study has several strengths, including the exposure to PUFA supplementation over the life-course, the balanced composition of the intervention diets to provide attainable dietary PUFA levels, and the longitudinal evaluation of in vivo bone microarchitecture using µCT. Although commonly used to test nutritional interventions [19], a limitation of this study involves the young age (3 months) of the rats when randomized to surgery as rats may still be undergoing skeletal growth [5] and the relatively small VOI used for the determination of body composition. A further limitation of this study was the inability to interpret some of the cortical bone changes. Although the µCT scans were completed at sufficient resolution to attain a high quality image [33], a longer follow-up period may have been necessary to observe changes in both primary and secondary outcomes.

In summary, providing a diet with varying n-6 to n-3 PUFA ratio at levels attainable through healthful diet patterns in humans does not cause consistent changes to the primary bone structure outcomes most commonly associated with changes to bone strength (i.e., BV/TV, Ct.Ar./Tt.Ar., Ct.Th.). Feeding a lower PUFA ratio (5:1 versus 10:1) by providing higher absolute amounts of n-3 (as flaxseed oil) does result in benefits to cortical bone during growth and these benefits are sustained during periods of bone remodeling (following OVX). However, feeding this diet (5:1 (flaxseed)) resulted in significant detriments to trabecular bone compared to 5:1 (menhaden) fed Sham groups. In conclusion, altering the source and absolute amount of n-3 PUFA to provide varying n-6 to n-3 PUFA ratios in the diet caused differences in bone structure between groups at 3 and 6 months of age but many of these differences are not consistent. Dietary n-3 PUFA
provided at two physiologically relevant levels is not an adequate strategy to protect against OVX-induced trabecular bone loss.
References


42. Goyens, P.L., Spilker, M.E., Zock, P.L., Katan, M.B., Mensink, R.P., Conversion of alpha-linolenic acid in humans is influenced by the absolute amounts of alpha-


Chapter 8: General Discussion
**Major Research Findings**

The studies of this thesis can be grouped into a) those that evaluated the method of µCT scanning for its use in characterizing the time course of OVX-induced changes to bone microarchitecture of the proximal tibia (Studies 1 through 3), and b) those that evaluated the impact of a nutritional intervention on bone health during periods of growth and following OVX (Study 4). The major findings from Studies 1 through 3 are summarized in Figure 8-1.

The first study presented in this thesis (Study 1, Chapter 5) was performed to investigate the time course of structural changes to the trabecular and cortical bone of the proximal tibia of female rats having undergone Sham- or OVX-surgery at 3 months of age. Findings from this study showed that rats randomized to receive OVX-surgery experienced characteristic declines in trabecular BMD, BV/TV, and Tb.N., and increases in Tb.Sp. from surgery at 3 months of age to 5 months of age. These changes were maintained until 6 months of age, suggesting a plateau of trabecular bone loss was reached by 5 months of age (2.25 months post-surgery). In contrast, Sham-control rats did not experience changes to trabecular BMD, BV/TV, or Tb.Sp., and Tb.N. only slightly declined from 4 to 5 months of age. Therefore, these results suggest that OVX at 3 months of age produces a characteristic decline in trabecular bone quantity and structure, as measured by µCT, similar to the early events reported in postmenopausal women (1).

The changes to trabecular bone microarchitecture of the proximal tibia of OVX rats can be confidently compared to Sham-operated rats, as trabecular bone microarchitecture of Sham rats remained relatively stable, with only minor fluctuations in
Tb.Th., Tb.N., and DA throughout the study period. These findings are in contrast to previous studies, which described a reduced osteoporotic response in Wistar rats undergoing OVX at 3 months of age compared to Sham (2). The authors of this study suggested that Sham rats were still undergoing growth at this age. The discrepancy between this and the current study may be due to the species of rat under investigation or the scanning parameters used. A major strength of the current thesis involved the repeated *in vivo* imaging of the same rat over time to provide a time course of changes to bone with natural aging (Sham) and in response to OVX.

OVX at 3 months of age did not produce a change in cortical bone of the proximal tibia as expected. Also, a comparison of the changes to cortical bone from OVX and Sham animals is difficult as Sham rats experienced changes that suggest continued growth. Sham-control rats experienced an increase in Ct.Ar./Tt.Ar., Ct.Th., Ct.Ar., and a decrease in Ps.Pm. and Ma.Ar. Therefore, the 3-month SD rat can be considered a good preclinical model for the study of therapeutic agents on the trabecular bone of the proximal tibia, but not the cortical bone. Food and Drug Administration guidelines do however recommend two different animal species to be tested, the second of which to be a larger OVX non-rodent species with more extensive cortical remodeling (3). Findings from Study 1 of this thesis are supportive of this recommendation, as the cortical bone outcomes tested experienced a great deal of fluctuation. This may be due to the age of the rat at OVX, but also because of the relatively low amount of intra-cortical remodeling experienced compared to human cortical bone (4). A disadvantage of the rat model involves its lack of Haversian systems (5, 6), which limit the surface area on which bone multicellular units initiate and complete remodeling cycles. More research should be
undertaken to investigate the changes to the cortical bone of the midpoint of the rat tibia from 3 through 6 months of age using *in vivo* µCT.

Current guidelines for the preclinical evaluation of drugs and other therapeutic agents for the treatment of osteoporosis provide recommendations for the animal models used, but fail to recommend the ages of the rats to be used (3). Several authors have suggested performing OVX at 9 months of age, when the rats near senescence (7-9), but ordering, aging, and maintaining laboratory rats for extended periods of time becomes costly and time-consuming. For this reason, many preclinical studies of osteoporosis drug therapies have used the younger, 3-month OVX rat model (10-12). *Findings from this thesis support the use of this model when assessing trabecular bone structure by µCT.*

A novel finding of Study 1, and arguably the most important, was the significant difference observed in BMD, BV/TV, and all other trabecular indices between Sham and OVX rats only one week post-surgery. This is the earliest detectable loss of bone reported in the literature. *This finding highlights the importance of attaining a true baseline value (before randomization to Sham or OVX), only possible by performing surgeries on-site and not by the supplier.*

Findings from Study 2 (Chapter 5) demonstrated that *repeated in vivo µCT scanning of the proximal tibia at intervals commonly used for the longitudinal evaluation of bone microarchitecture does not affect the trabecular or cortical outcomes examined.* The only main effect of repeated radiation at 600 mGy was a decrease in Ecc., describing the change in the cortical bone shape from circular toward elliptical. Changes to this parameter are often associated with altered load bearing. Although a strength of this
study was the use of contralateral limbs to reduce any inter-animal variability, the loads applied to the ankle and distal tibia during repeated scanning were not controlled for and may have caused the differences in Ecc. that were observed. A similar finding of decreased Ecc. with repeated irradiation has been found in our lab using a male mouse model at a radiation dose of 222 mGy (Sacco et al. 2016, unpublished data), further suggesting that this finding was not due to chance, alone. Results from Study 2 provide future researchers with confidence when interpreting results of an intervention without the confounding effects of radiation exposure. This is critical as the use of µCT for the assessment of bone quality is the gold standard in preclinical rodent studies (13-15).

Following the completion of Study 1 and 2, Study 3 (Chapter 6) was designed to determine the relative impact of µCT scanning parameters on the trabecular and cortical outcomes of the tibia. *Results from this study demonstrated that in vivo and ex vivo scanning of the proximal tibia in Sham or OVX rats did not affect the trabecular or cortical outcomes assessed.* This finding is important for the design of future experiments. Considerations must be made when designing experiments with preclinical rodent models for the time and cost of many endpoint measures. While utilizing more animals provides a more comprehensive assessment of changes to bone health, the feasibility of scheduling these *in vivo* measurements is not always practical. With the results from Study 3, researchers can confidently exchange the timely and costly endpoint *in vivo* µCT scans with *ex vivo* scans of harvested and cleaned bone samples, to be completed at a later, more convenient time. And, these *ex vivo* scans can be compared to *in vivo* scans of baseline and interim time points with confidence. While this study only assessed a single skeletal site, future research should be undertaken to determine if
quantification of bone quality at other sites, such as the LV, are also unaffected by scan parameters. Results of this study cannot simply be extrapolated to these other bone sites because a thin layer of soft tissue surrounds the proximal tibia only, whereas a thick layer of muscle, adipose, skin, and organs surrounds the LV. This surrounding tissue alters the path of the X-ray, and may ultimately have an effect on the outcomes assessed.

Scanning of the tibia *ex vivo* at a voxel size of 18µm over or underestimated several of the trabecular and cortical outcomes that are quantified, due to the limited extent to which the bone network can be visualized compared to scans of 9µm. This finding is important when interpreting and comparing absolute values among studies (or within studies) using different scanning techniques and voxel sizes. The best evidence of this is demonstrated in the shifted distribution of Tb.Th. between 18µm and 9µm scan settings (Figure 6-1). When comparing relative values however, most microarchitecture outcomes were correlated between 9µm and 18µm scan settings. This supports the use of a larger voxel size when assessing the relative changes at the proximal tibia between groups in a study. This is applicable to studies of very large sample size as smaller voxel size scans require significantly more time and hard drive space when scanning, reconstructing, and analyzing.
Use of µCT for the characterization of OVX rat model of postmenopausal osteoporosis

<table>
<thead>
<tr>
<th>Study 1</th>
<th>Study 2</th>
<th>Study 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham rats experienced:</td>
<td>• Repeated radiation exposure down Ecc.</td>
<td>• No effect of in vivo versus ex vivo scanning</td>
</tr>
<tr>
<td></td>
<td>Trabecular Outcomes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• No change in BMD, BV/TV, Tb.Sp.</td>
<td>• Repeated radiation exposure did not affect any other trabecular or cortical outcome of the proximal tibia</td>
</tr>
<tr>
<td></td>
<td>• ↓ Tb.N., DA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• Fluctuation in Tb.Th.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cortical Outcomes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>• ↑ Ct.Ar./Tt.Ar., Ct.Th.</td>
<td>• Smaller sample size needed to detect differences between Sham and OVX groups with in vivo scanning</td>
</tr>
<tr>
<td></td>
<td>• ↓ Ps.Pm., Ma.Ar.</td>
<td>• Repeated radiation exposure did not affect any other trabecular or cortical outcome of the proximal tibia</td>
</tr>
<tr>
<td></td>
<td>• Fluctuation in Ec.Pm., Ecc.</td>
<td>• No effect of in vivo versus ex vivo scanning</td>
</tr>
<tr>
<td>OVX rats experienced:</td>
<td>• ↓ BMD, BV/TV, Tb.N.</td>
<td>• Smaller sample size needed to detect differences between Sham and OVX groups with in vivo scanning</td>
</tr>
<tr>
<td>Trabecular Outcomes</td>
<td>• ↑ Tb.Sp.</td>
<td>• Repeated radiation exposure did not affect any other trabecular or cortical outcome of the proximal tibia</td>
</tr>
<tr>
<td></td>
<td>• Fluctuation in Tb.Th., DA</td>
<td>• Smaller sample size needed to detect differences between Sham and OVX groups with in vivo scanning</td>
</tr>
<tr>
<td>Cortical Outcomes</td>
<td>• ↓ Ec.Pm., Ma.Ar.</td>
<td>• Scanning with different voxel sizes alters absolute but not relative differences between groups</td>
</tr>
<tr>
<td></td>
<td>• ↑ Ct.Ar./Tt.Ar., Ct.Th.</td>
<td>• All scan parameters tested are able to determine differences between Sham and OVX groups</td>
</tr>
<tr>
<td></td>
<td>• Fluctuation in Ps.Pm., Ecc.</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 8-1. Summary of the major research findings from Study 1, 2, and 3**

These studies evaluated the method of µCT scanning for its use in characterizing the time course of bone microarchitecture changes of the proximal tibia following OVX at 3-months of age.
Study 1 to 3 of this thesis were methodological in nature and further characterize the use of *in vivo* and *ex vivo* imaging of preclinical models of osteoporosis by μCT. Results of these methodological studies influenced the experimental design of Study 4 (Chapter 7) in several ways: providing a time course for the changes to bone structure following Sham and OVX surgery at 3 months of age, providing confidence in the method and safety of repeated in vivo scanning of the proximal tibia, highlighting the necessity of conducting Sham and OVX surgeries independently, and refining the scan settings used to provide high image quality.

A summary of the major research findings from Study 4 is reported in Figure 8-2. *Results of this nutritional intervention demonstrate that a PUFA intervention of varying n-3 source and n-6 to n-3 ratio can modulate bone microarchitecture during periods of rapid growth, but that this may not be an adequate strategy for the prevention and treatment of OVX-induced bone loss.* Feeding diets of 5:1 (Flaxseed) from 1 through 3 months of age reduced Tb.Th., and Ma.Ar. and increased Ct.Ar./Tt.Ar. compared to rats receiving diets with a 10:1 PUFA ratio in the proximal tibia. These changes to the cortical bone in young, growing rats are suggestive of a response to consuming a lower PUFA ratio when n-3 is provided primarily as ALA from flaxseed oil, but not as EPA and DHA from menhaden oil. At 6 months of age, an effect of diet was observed in only some cortical indices. To determine the effect of diet and hormone status following randomization to Sham or OVX surgery only, the change in microarchitecture outcomes from 3 through 6 months of age can be quantified in each animal. This analysis removed any initial effect of diet on bone outcomes from 1 through 3 months of age. When the change from 3 to 6 months of age was analyzed, diet had a significant effect on cortical
BMD and Ecc. only. Together, these results suggest that bone of the proximal tibia is responsive to PUFA intervention (specifically when supplied in a 5:1 ratio with n-3 provided as ALA from flaxseed oil) during periods of rapid growth, but that these changes are not maintained following Sham or OVX surgery.

When LV4 was analyzed in a subset of rats at 3 months of age (n=4-6/group), fewer main effects of diet were uncovered, highlighting the skeletal-site specific response to an intervention in the rat. This is most likely due to an altered loading pattern and the mechanical forces that are applied to the proximal tibia compared to the LV4 in the rat. Rats are quadrupeds, and therefore their spines experience less loading than bipeds. Of importance to note, LV4 analysis was done ex vivo. Following from results from Study 3, the removal of soft tissue surrounding LV3 may have had an effect on the quantification of bone outcomes. Also, the exposure of the organs and abdominal tissues to the radiation exposure necessary to acquire a high quality image was a concern and prevented in vivo measurements at this skeletal site.
Use of μCT for the evaluation of lifelong PUFA-supplementation

<table>
<thead>
<tr>
<th>Growth (1-3 months)</th>
<th>Following SHAM/OVX</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proximal Tibia:</strong></td>
<td><strong>Proximal Tibia:</strong></td>
</tr>
<tr>
<td>• 5:1 (Flaxseed) ↓ Tb.Th., ↑ Ct.Ar./Tt.Ar. (compared to 10:1 groups)</td>
<td>• 10:1 (Flaxseed) ↓ cBMD (compared to all groups)</td>
</tr>
<tr>
<td>• 5:1 (Flaxseed) ↓ Ma.Ar. (compared to 10:1 (Flaxseed))</td>
<td>• 5:1 (Flaxseed) ↑ Ct.Ar./Tt.Ar., ↓ Ma.Ar.</td>
</tr>
<tr>
<td><strong>LV4:</strong></td>
<td>Serum Biomarkers:</td>
</tr>
<tr>
<td>• 10:1 (Menhaden) ↓ DA (compared to Flaxseed groups)</td>
<td>• No effect of diet on OPG, RANKL, or their ratio</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Change from 3-6 months</th>
<th><strong>Proximal Tibia:</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proximal Tibia:</strong></td>
<td>• 5:1 (Menhaden) ↑ tBMD, BV/TV, Tb.N., ↓ Tb.Sp. (compared to 5:1 (Flaxseed))</td>
</tr>
<tr>
<td>• 10:1 (Flaxseed) ↓ cBMD (compared to all groups)</td>
<td><strong>LV4:</strong></td>
</tr>
<tr>
<td></td>
<td>• 5:1 (Menhaden) ↑ Tb.N. (compared to 10:1 (Flaxseed))</td>
</tr>
</tbody>
</table>

**Trabecular Outcomes**

**Proximal Tibia:**
- 5:1 (Menhaden) ↑ tBMD, BV/TV, Tb.N., ↓ Tb.Sp. (compared to 5:1 (Flaxseed))

**LV4:**
- 5:1 (Menhaden) ↑ Tb.N. (compared to 10:1 (Flaxseed))

**Cortical Outcomes**

**Proximal Tibia:**
- 5:1 (Menhaden) ↓ Ct.Ar., Ma.Ar. (compared to 10:1 (Flaxseed))
- 5:1 (Menhaden) ↑ Ct.Th. (compared to all diets)
- 5:1 diets ↓ Ct.Ar./Tt.Ar. (compared to 10:1 diets)

Figure 8-2. Summary of the major research findings from Study 4
Because of the robust effect of OVX on the microarchitecture of bone, Sham and OVX rats were examined independently. When this was done, Sham and OVX rats experienced diet-related changes to trabecular and cortical bone, respectively. Benefits were observed to bone health in both groups when rats received diets with a low PUFA ratio (5:1 compared to 10:1). The finding that different bone types responded to diet in different surgery-type rats is most likely because of the bone that was available for the nutrients to influence. Following OVX or menopause, trabecular bone is lost more rapidly, not because of its composition, but because of its increased surface area on which bone multicellular units can be initiated (16). Therefore, in the 3-months post-OVX, the nutritional intervention that rats were randomized to is only able to influence cortical bone structure, as almost all trabeculae have been resorbed. This finding strongly suggests against the adequacy of a nutritional intervention, specifically dietary PUFA, to protect against such drastic and deleterious trabecular bone loss induced by OVX.

Study 4 also examined the effect of diet and hormone status on body composition using in vivo µCT. In contrast to many findings reported in the literature, hormone status did not have an effect on lean, non-lean, or bone mass. Following OVX, rats experience significant increases in fat mass compared to their Sham counterparts (17). This discrepancy is likely due to the relatively small ROI under investigation. Body composition was measured in the abdominal region surrounding LV3 only, a relatively small region compared to length of multiple vertebrae when assessed by DXA (17) and in validation studies of µCT (18-20). Diet did however have an effect on body composition. Within Sham rats, those receiving diets of 5:1 (Menhaden) had lower percent lean mass volume compared to all other Sham rats and greater non-lean volume relative to VOI
length compared to 5:1 (Flaxseed) fed Sham rats. This result is in line with the findings in bone, that ALA fed groups experience greater benefits to health, including lower non-lean (fat) mass and greater lean mass compared to fish oil fed rats.

This intervention study also examined bone biomarkers in an effort to determine the mechanism by which bone structure is changing. As expected, OVX significantly increased serum RANKL, lower serum OPG and the ratio of OPG to RANKL compared to Sham rats (21-23), but there was no effect of diet on these markers. The lack of findings may be due to the timing of blood collection, as treatment-induced effects on markers of bone turnover occur rapidly in rat blood and skeletal tissue (21, 24, 25). Limited studies in cell culture models have suggested that n-6 PUFA stimulate osteoclastogenesis and downstream bone resorption through the OPG/RANKL pathway (26, 27), but no studies have investigated the role of ALA on the expression of OPG and RANKL or in comparison with the longer chain n-3 PUFA. While findings from this thesis do not suggest that the changes to bone structure are through altered expression of OPG and RANKL, studies in cell culture should be undertaken before this mechanism is dismissed.

**Implications for Future Research using μCT**

The findings from this thesis highlight the safety of repeated *in vivo* μCT scanning of the hind limb of the rat at a monthly radiation exposure of 600 mGy. This amount of X-ray radiation, applied directly to the hind limb, can be attained with settings necessary for the acquisition of high-quality images and appropriate X-ray transmission levels. The hormone status (Sham versus OVX) of the rat does not alter its resistance to repeated X-ray radiation.
When examining the proximal tibia, *in vivo* and *ex vivo* scanning does not affect the trabecular or cortical outcomes assessed. Scanning the proximal tibia at a voxel size of 18µm does however significantly change the absolute values of some microarchitecture outcomes compared to scanning at 9µm voxel size. Scanning at these voxel sizes do not result in relative differences. Therefore, timely and costly endpoint *in vivo* scanning can be replaced with *ex vivo* scanning in future experiments. Also, relative (but not absolute) values can be compared across studies of different voxel size and scan settings.

**Implications for Future Research using the OVX Rat Model of Postmenopausal Osteoporosis**

The findings from this thesis provide a basis for the further use of the 3-month OVX SD rat as a model of postmenopausal osteoporosis. *This rat model can be used for the assessment of changes to trabecular bone of the proximal tibia.* Trabecular bone of the 3-month OVX rat experiences characteristic declines in BMD, bone volume, and trabecular microarchitecture indices over a 3-month time course. While Sham-operated rats do not experience changes to the trabecular bone microarchitecture from 3 through 6 months of age, suggesting these rats do not experience substantial growth between these time points. *This model should not be used for the assessment of changes to cortical bone at this skeletal-site.* Further study should be undertaken to determine if the diaphysis or midpoint of the tibia or femur is a more appropriate skeletal-site when analyzing cortical bone.

The applicability of this model to the menopausal transition in women should however be strongly considered when designing future experiments. Unlike the human
condition, OVX causes a severe and abrupt end to endogenous hormone production with the complete removal of the ovaries. While the natural transition to menopause in humans is experienced over a period of several years. With this slowed loss of estrogen production, women experience a ‘window of opportunity’ during the 5-10 year period coinciding with the transition in which bone turnover is greatly up-regulated and bone cells may be more responsive to treatment (28, 29). To appeal this difference when designing preclinical studies, the intervention should be of sufficient duration to be equivalent to a one-year study in humans. The average bone turnover cycle in humans and rats is roughly 200 and 40 days, respectively (30, 31). Therefore, a 12-week study in the rat is approximately two complete remodeling cycles, which is roughly equivalent to a 1.5-year trial in humans.

Dietary PUFA as Prevention for Postmenopausal Osteoporosis

In Animals

Results from this thesis (Study 4, Chapter 7) are not supportive of a dietary PUFA intervention of varying PUFA ratio and n-3 source as a prevention of OVX-induced bone loss. Although varying the PUFA ratio and n-3 source to levels attainable in the human diet through healthful nutrition patterns altered aspects of bone structure during periods of rapid growth, these changes were not sustained following OVX.

These findings support the notion that nutrients are not drugs, and at levels attainable through diet alone and not with supplementation, do not act on the skeleton like drugs. This is especially true after the abrupt hormonal-insult of OVX. These conclusions however, are not a cause for concern, or a reason to abandon all healthful eating habits. As summarized in Chapter 2, there are several studies in the literature supporting the intake of a lower PUFA ratio and n-3’s from either plant or fish oils on
bone health outcomes in a preclinical model. Not surprisingly, these positive findings are often observed when an intervention diet is compared to a control diet with very high n-6 content (i.e., safflower oil-fed control, corn oil-fed control) and a very high PUFA ratio. A major strength of this thesis (Study 4) was the composition of the four intervention diets, and their physiologically relevant PUFA ratios. If a 5th diet were to be included as an n-6 ‘control diet’, it is hypothesized that all PUFA intervention diets would have significantly improved all bone-related outcomes by comparison. This was not the objective of the current study, however, as we aimed to determine changes to bone health with PUFA levels attainable through diet alone. While PUFA-intervention alone was not an adequate strategy for the prevention of OVX-related trabecular bone loss, flaxseed has been shown to improve bone mass and strength in combination with low dose estrogen (32). Therefore, PUFA may be a novel pairing in future research of food-drug synergy and the prevention of postmenopausal osteoporosis.

A major strength of this thesis was the study of exposure to PUFA interventions over the life course and the repeated in vivo imaging of the proximal tibia throughout periods of growth and following OVX. Unlike studies of a nutritional intervention introduced immediately following OVX, this design applied to human diet patterns, which remain relatively constant throughout the lifespan (33, 34). Therefore, the nutritional intervention was tested as a preventative approach, rather than a treatment. And unfortunately, a lifelong PUFA intervention does not protect against trabecular bone loss induced by OVX at 3 months of age in the rat model.

**In Humans**
Worldwide, the incidence of fragility fracture varies substantially and this variation may be attributable to difference in lifestyle, including dietary patterns (35). The relation between PUFA intake and aspects of bone health have been tested in several observational studies and RCTs of postmenopausal women and in general, have yielded strong associations between some PUFAs and bone health in the observational studies, but not with the RCTs (Table 2-12).

There are several reasons that positive findings have been found with observational studies, but null effects have been reported in RCTs. First, the diet pattern of an individual remains fairly stable throughout the lifespan (33, 34). Therefore, when assessing dietary patterns through observational studies, the nutrient under investigation has existed in the individual, having its biological effect, for years at a time. This major difference between observational studies and RCTs was taken into consideration when designing Study 4, aimed at determining the effect of PUFA supplementation over the life course in the rat. Furthermore, RCTs assessing bone health outcomes in response to a change in PUFA intake have generally resulted in a null effect because the experiment follows a design specifically developed for the testing of drugs (36) and as mentioned above, nutrients are not drugs.

To begin, no RCTs reviewed in this thesis measured the basal PUFA status, although some studies did exclude participants based on consumption of flaxseed products, n-3 supplements, or high intakes of fish (37-39). It is important to have an understanding (or a nominal value) of a participant’s baseline nutritional status, because this will change the response of the nutrient under investigation. For example, if the participant is deficient or consuming lower than recommended levels of a nutrient or food
component, supplementation will produce a measurable benefit, whereas, if the participant is replete, supplementation will produce a null effect. Similarly, the nutritional intervention being tested must be large enough to effectively change the nutrient status. RCTs of ALA supplementation reviewed in this thesis have provided between 5.75 – 17.3 g ALA/d as flaxseed with no observed benefit to bone health (37, 38). Rather than assuming no benefit to bone occurs with ALA supplementation in postmenopausal women, the nutrient range that provides a benefit may have been exceeded. This is supported by prospective studies reporting a decrease total fracture risk and increase lumbar spine BMD with increasing ALA intake above 0.08 g ALA/d and above 1.02 g ALA/d, respectively (40, 41). Therefore, what may be more pertinent to our understanding of ALA (and EPA+DHA) are studies intended to quantify the transition between inadequate to adequate nutrient status and the relevant range of intake necessary for a response.

Finally, and arguably most difficult, when testing the effect of specific nutrients, co-nutrient status must also be considered and controlled for. Nutrients interact with one another (i.e., PUFA ratio, calcium and vitamin D) therefore, all other interacting nutrients should also be considered. Specifically, in studies of bone health, it is important to control for calcium, vitamin D, and protein intake. Failure to control for these other bone-supporting nutrients may influence individual results, and the overall findings of a study.
Chapter 9: Conclusions
Conclusions

Study 1: Time-course of the changes to bone microarchitecture in the 3-month OVX rat model of postmenopausal osteoporosis

- Randomization to surgery at 3-months of age resulted in characteristic declines in trabecular and cortical BMD, BV/TV, Tb.N., and Ec.Pm., and increases in Tb.Sp. in OVX rats but not in Sham-control rats.
- Sham rats experienced decreased Ps.Pm., and Ma.Ar.
- OVX rats experience decreased Ec.Pm., Ma.Ar., and increased Ct.Ar./Tt.Ar., and Ct.Th.

Study 2: Characterization of the effect of repeated radiation exposure by in vivo µCT scanning in rat tibia

- Monthly repeated radiation exposure did not affect the trabecular microarchitecture of the proximal tibia in Sham or OVX rats.
- Monthly repeated radiation exposure reduced mean Ecc. of the proximal tibia. No other cortical microarchitecture outcome was affected by repeated radiation exposure in Sham or OVX rats.

Study 3: Comparison of ex vivo and in vivo µCT of rat tibia at different scanning settings

- Scanning of the proximal tibia in vivo or ex vivo did not affect the quantification of trabecular or cortical microarchitecture in Sham or OVX rats.
- Scanning of the proximal tibia at 18µm voxel size overestimated Tb.Th., Ct.Ar., Ct.Th., DA, Ct.Ar./Tt.Ar., and underestimated Conn.D. compared to 9µm voxel size scans.
- Regardless of scan protocol, significant differences between Sham and OVX rats can be detected for all microarchitecture outcomes, expect Tb.Th.
Study 4: Lifelong intake of flaxseed or menhaden oil to provide varying n-6 to n-3 PUFA ratios modulate bone microarchitecture during growth, but not after OVX

- Feeding ALA from flaxseed in a 5:1 ratio from 1 through 3 months of age resulted in decreased Tb.Th., Ma.Ar., and increased Ct.Ar./Tt.Ar. of the proximal tibia and increased DA of the LV4.
- Diet had no effect on the trabecular bone microarchitecture of the proximal tibia at 6 months of age or when the change from 3 to 6 months of age was assessed.
- Within Sham rats, feeding EPA+DHA from menhaden oil in a 5:1 ratio favourably altered some trabecular bone outcomes compared to 5:1 (Flaxseed) fed rats.
- Within OVX rats, diet had an effect on cortical but not trabecular microarchitecture.
Chapter 10: Future Research
Future Research

The results of this thesis have identified a number of areas for future research. Findings from Study 1 do not support the use of the cortical bone of the proximal tibia as an appropriate model for use in drug and nutrition therapies, but an investigation into the time course of changes to the midpoint of the tibia in the 3-month OVX rat model should be undertaken. At the midpoint, high-resolution imaging can be used to quantify cortical porosity and changes to intra-cortical remodeling. As the guidelines for designing preclinical studies to support the approval of therapies intended for the treatment of osteoporosis suggests, a time course of changes to bone microarchitecture should be completed in a second, larger non-rodent species with more extensive cortical remodeling, similar to in humans.

Further research should also be conducted to test the effect of scanning in vivo compared to ex vivo at a second skeletal site, such as the LV. Results from Study 3 cannot simply be extrapolated to this site that is surrounded by significantly more soft tissue. Similarly, the effect of repeated radiation exposure to the organs surrounding the LV should be elucidated to determine the safety of ionizing radiation to this region of the body.

Although the OVX rat experiences changes to bone quantity and quality that are characteristic of postmenopausal women, the development and use of a preclinical model that more closely mimics the human situation during the menopausal transition is an area for future research. Unlike in humans, OVX causes an abrupt end to endogenous hormone production, and a substantial and rapid decline in skeletal quantity and quality. Future investigation should be focused on the development of a model that is still
relatively inexpensive, can be studied over the short-term, has a similar skeletal anatomy to humans, and can be raised in standardized conditions, but undergoes a steady transition from pre- to post-menopause. With a model that more closely mimics the human condition, whether PUFA-supplementation over the life course more effectively protects against the hormone-induced bone loss can be determined. Such a model would also allow us to identify other dietary modulations that promote and support bone health during the menopausal transition and the ‘window of opportunity’.

As seen in Study 4, supplementation with a lower PUFA ratio when n-3 was provided as ALA from flaxseed oil resulted in changes to the growing skeleton, potentially increasing peak bone mass in the rat. Although this did not result in any protection against the abrupt hormonal insult of OVX, whether long-term intake of a similar PUFA profile can increase peak bone mass in young adults and protect against the natural loss of bone in the postmenopausal situation is a novel research question. Moreover, the mechanism(s) by which ALA and EPA+DHA exert their effects on bone health during periods of rapid growth are an area of future research needed to further our understanding. With knowledge of the mechanism of action of these n-3 PUFA, food-drug synergy experiments can be explored as potential therapies for the prevention and protection against the development of postmenopausal osteoporosis.
References


