FLAXSEED AND LOWER-DOSE ESTROGEN: STUDIES ON THEIR PROTECTIVE ACTIONS AND MECHANISMS IN BONE USING THE OVARIECTOMIZED RAT MODEL

by

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A thesis submitted in conformity with the requirements for the degree of Doctorate of Philosophy

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Abstract

Flaxseed (FS) is a rich source of lignans and n-3 polyunsaturated fatty acids (PUFA), compounds that may help preserve normal bone cell function during aging. Understanding the effect of FS alone or combined with lower doses of estrogen therapy on bone and other estrogen-responsive tissues (e.g. uterus) is of particular interest to postmenopausal women who combine dietary bioactives (e.g. FS) with pharmacological agents (e.g. estrogen monotherapy) to attenuate postmenopausal bone loss. The overall objective of my research was to determine the effects and mechanisms of FS, alone or combined with lower doses of estrogen therapy, on bone and uterus health by measuring a comprehensive set of outcomes in the ovariectomized rat model of postmenopausal osteoporosis. The results demonstrated that FS enhances the protective effect of low-dose estrogen therapy (LD) on vertebral bone mineral density (BMD), three-dimensional microarchitecture and strength in ovariectomized rats. Moreover, FS exerts a stronger effect on bone outcomes when combined with LD than when combined with ultra-low-dose estrogen therapy (ULD). These studies also showed that FS feeding results in higher lignans and n-3 PUFAs in vertebrae, tibias and femurs. Histological analyses at the lumbar vertebra (LV) showed that there were no differences in TRAP-5β, CTX, or OPG/RANKL ratio between the
FS+LD and LD groups. FS+LD did however result in lower protein expression of osteocalcin, a marker of bone formation and overall bone turnover, and higher expression of OPG compared to the negative control (NEG), while LD did not. While these findings suggest that FS+LD results in greater attenuation of deterioration of bone tissue compared to LD due to a reduction in bone turnover, significant differences between FS+LD and LD were not observed. Elucidating these specific mechanisms of action require further investigation. In the uterus, FS+LD did not induce greater cell proliferation or differences in qualitative indices of uterine morphology compared to LD. These findings suggest that there may be no increase in the risk of endometrial hyperplasia and carcinoma with FS+LD compared to LD. These findings may lead to the development of strategies that combine food bioactives and current pharmacological agents to more effectively normalize bone turnover during aging.
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<th>Definition</th>
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<tbody>
<tr>
<td>ALA</td>
<td>α-linolenic acid</td>
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<tr>
<td>BMC</td>
<td>bone mineral content</td>
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<tr>
<td>BMD</td>
<td>bone mineral density</td>
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<tr>
<td>BMU</td>
<td>basic multicellular unit</td>
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<tr>
<td>CEE</td>
<td>conjugated equine estrogen</td>
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<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CTX</td>
<td>C-terminal telopeptides of type I collagen</td>
</tr>
<tr>
<td>DEXA</td>
<td>dual energy x-ray absorptiometry</td>
</tr>
<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
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<tr>
<td>ED</td>
<td>enterodiol</td>
</tr>
<tr>
<td>EL</td>
<td>enterolactone</td>
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<tr>
<td>EPA</td>
<td>eicosapentaenoic acid</td>
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<tr>
<td>ER</td>
<td>estrogen receptor</td>
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<tr>
<td>ERα</td>
<td>estrogen receptor alpha</td>
</tr>
<tr>
<td>ERβ</td>
<td>estrogen receptor beta</td>
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<tr>
<td>FDA</td>
<td>United States Food and Drug Administration</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>FS</td>
<td>flaxseed</td>
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<tr>
<td>HERS</td>
<td>Heart and Estrogen/Progestin Replacement Study</td>
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<tr>
<td>HOPE</td>
<td>Women’s Health, Osteoporosis, Progestin, Estrogen Trial</td>
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<tr>
<td>HR</td>
<td>hazard ratio</td>
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<tr>
<td>HT</td>
<td>hormone therapy</td>
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<td>IGF-1</td>
<td>insulin-like growth factor 1</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
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<tr>
<td>LD</td>
<td>low-dose</td>
</tr>
<tr>
<td>LV</td>
<td>lumbar vertebra</td>
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<tr>
<td>M-CSF</td>
<td>macrophage colony-stimulating factor</td>
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<tr>
<td>MP</td>
<td>micronized progesterone</td>
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<tr>
<td>MPA</td>
<td>medroxyprogesterone acetate</td>
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<tr>
<td>NA</td>
<td>norethisterone acetate</td>
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<tr>
<td>NEG</td>
<td>negative control</td>
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<td>NF-κB</td>
<td>Nuclear Factor kappa Beta</td>
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<td>NTX</td>
<td>N-terminal peptides of type I collagen</td>
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<td>OPG</td>
<td>osteoprotegerin</td>
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<td>OVX</td>
<td>ovariectomized</td>
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<tr>
<td>PEPI</td>
<td>Postmenopausal Estrogen/Progestin Interventions Trial</td>
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<tr>
<td>PGE₂</td>
<td>series 2 prostaglandins</td>
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<tr>
<td>PND</td>
<td>postnatal day</td>
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<td>POS</td>
<td>positive control</td>
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<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acids</td>
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<tr>
<td>RANK</td>
<td>receptor activator of NF-κB</td>
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<tr>
<td>RANKL</td>
<td>RANK ligand</td>
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<tr>
<td>RCT</td>
<td>randomized control trial</td>
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Runx2: runt-related transcription factor 2
SDG: secoisolariciresinol diglycoside
SOF: study of osteoporotic fractures
TGF-β: transforming growth factor beta
TNF-α: tumor necrosis factor alpha
TRAP: tartrate-resistant acid phosphatase
ULD: ultra-low-dose
ULTRA: Ultra-Low-Dose Transdermal Estrogen Assessment Trial
WHI: Women’s Health Initiative Trial
WHO: World Health Organization
WISDOM: Women's International Study of long Duration Oestrogen after Menopause
CHAPTER 1
Introduction
1.0 Introduction

Osteoporosis is a skeletal disease characterized by compromised bone strength, predisposing an individual to an increased risk of fractures (NIH Consensus Development Panel on Osteoporosis Prevention Diagnosis and Therapy, 2001). The rapid decline in endogenous estrogen production that occurs during menopause results in a significant increase in bone turnover such that the increased rate in bone resorption outweighs the increased rate of bone formation. Consequently, menopause results in significant bone loss and increased risk for fragility fracture.

While estrogen therapy is an effective means to attenuate bone turnover and the risk of future fracture, reported side effects associated with its use has resulted in a rapid decline in its prescription and use worldwide (Hersh et al., 2004; Burge et al., 2007). However, significant limitations to study design, particularly in the Women’s Health Initiative (WHI) (Rossouw et al., 2002; Anderson et al., 2004), have become the focus of subsequent dialogue, stimulating the investigation of lower doses of estrogen therapy using a transdermal delivery system to determine whether they can protect against postmenopausal bone loss while inducing fewer side effects than standard dose therapies (Ettinger, 2007). Recent findings demonstrate that lower doses of estrogen therapy through the transdermal delivery system attenuates postmenopausal bone loss and may induce fewer side effects than the standard dose oral therapy (Peeyananjarassri & Baber, 2005). In addition, since the observed side effects in the WHI were greater in women with an intact uterus using combination therapy (conjugated equine estrogen + medroxyprogesterone acetate [CEE+MPA]) than in hysterectomized women using monotherapy (CEE alone), lower doses of transdermal estrogen monotherapy are now also being tested on women with an intact uterus because it is believed that they will induce fewer side effects than the standard CEE+MPA therapy. Indeed, lower doses of transdermal estrogen monotherapy may
be prescribed to both hysterectomized and non-hysterectomized women without the concurrent administration of progesterone.

The use of natural health products has become increasingly common in North American women for the management of menopausal symptoms including bone loss (Chong et al., 2007) because they are often regarded as safer therapies compared to pharmacological methods. Flaxseed (FS) is an oilseed commonly consumed in the Western diet and is a rich source of lignans and n-3 polyunsaturated fatty acids (PUFA), components that can modulate bone metabolism (Ward et al., 2001b; Ward et al., 2001a; Kim et al., 2002; Mollard et al., 2005; Griel et al., 2007; Power et al., 2007; Boulbaroud et al., 2008; Feng et al., 2008). Secoisolariciresinol diglycoside (SDG), the most abundant lignan in FS, is metabolized by colonic bacteria into the more biologically active mammalian lignans, enterodiol (ED) and enterolactone (EL) (Thompson et al., 1991; Wang et al., 2000). Because the mammalian lignans share structural similarity to the steroid hormone 17β-estradiol, they can elicit weak estrogenic or antiestrogenic action in hormone-sensitive tissues (Penttinen et al., 2007; Penttinen-Damdimopoulou et al., 2009) and have the potential to modulate hormone-related diseases such as osteoporosis. Alpha-linolenic acid (ALA), an n-3 PUFA that is found in high quantity in FS, may also have the potential to modulate osteoporosis as diets high in ALA have been shown to exert favorable effects on markers of bone turnover and bone strength in ovariectomized mice and rats (Power et al., 2007; Boulbaroud et al., 2008). ALA is also metabolized in small amounts into long-chain PUFA including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Plourde & Cunnane, 2007). Although little is known regarding the mechanisms of action of n-3 PUFA, particularly ALA, on bone metabolism, it is generally proposed that, like 17β-estradiol, n-3 PUFA can attenuate the loss of bone mineral and bone strength by inhibiting the local synthesis of pro-inflammatory mediators (e.g. prostaglandin E₂ (PGE₂), tumor necrosis factor-α (TNF-α)) that are
involved in stimulating osteoclastogenesis and subsequent bone loss (Sakaguchi et al., 1994; Watkins et al., 2006).

Some (Ward et al., 2001b; Griel et al., 2007; Power et al., 2007; Boulbaroud et al., 2008; Feng et al., 2008) but not all (Ward et al., 2001a; Lucas et al., 2002; Brooks et al., 2004; Dodin et al., 2005; Mollard et al., 2005) studies have shown that FS and its components can favorably modulate bone metabolism but their potential effects in a postmenopausal osteoporotic situation have not yet been examined. In addition, dietary FS does not seem to stimulate non-skeletal tissues such as the uterus (Power et al., 2007; Chen et al., 2009; Penttinen-Damdimopoulou et al., 2009; Simbalista et al., 2010) suggesting that its consumption does not increase the risk of endometrial cancer, a notable side-effect associated with the use of standard-dose estrogen monotherapy. It is unknown, however, if FS can enhance or interfere with lower doses of transdermal estrogen therapy on attenuating postmenopausal bone loss without producing negative side effects in the uterus. If the combination of FS and lower doses of estrogen therapy are more effective than lower doses of estrogen therapy alone, it may be possible to develop diet-drug strategies to more effectively normalize bone cell metabolism during aging.

Because the lignans, n-3 PUFA and estrogen therapy may modulate bone metabolism through similar mechanisms of action, the overall hypothesis is that FS will protect against deterioration of bone tissue and will enhance the protective effect of lower doses of estrogen therapy by attenuating the rate of bone turnover while exerting no adverse effects on uterus health in the ovariectomized rat model of postmenopausal osteoporosis. The overall objective is to determine the effects and mechanisms of FS, alone or combined with lower doses of estrogen therapy, on deterioration of bone tissue and uterus health by measuring a comprehensive set of outcomes in the ovariectomized rat model of postmenopausal osteoporosis.
CHAPTER 2
Literature Review

Modified from:


2.0 Literature Review

2.1 Physiology of Bone Turnover

The skeleton is composed by weight of approximately 60% inorganic matter, 30-32% organic matter and 8-10% water (Gong et al., 1964). The inorganic phase consists of hydroxyapatite while the organic phase is composed of primarily type I collagen (98% of the organic phase) and various noncollagenous proteins (2% of the organic phase) such as osteocalcin, osteopontin, bone sialoprotein, a variety of enzymatic matrix modifiers (e.g. matrix metalloproteinases) and growth factors (e.g. transforming growth factor-β (TGF-β), insulin-like growth factor-1 (IGF-1), fibroblast growth factors (FGFs)) (Einhorn, 1994; Morgan et al., 2008). The material that makes up the inorganic and organic phases results in a dynamic tissue that serves a number of structural (e.g. support, mobility, protection to internal organs) and metabolic (e.g. regulation of mineral homeostasis, provision site for hematopoiesis) functions in the body. To fulfill these functions throughout the lifetime, bone is in a continuous process of remodeling (bone turnover) (Figure 2-1).

The remodeling cycle in the human skeleton occurs over a period of three to six months by the coordinated action of osteoclasts and osteoblasts, bone cells that are responsible for the resorption and formation of bone, respectively (Figure 2-1). Cortical bone, which makes up 80% of the skeletal mass and is found predominantly in long bones such as the femur, tibia, humerus, radius and ulna, has a rate of remodeling of approximately 2-5% per year in the adulthood and elderly (Bell et al., 1967; Hadjidakis & Androulakis, 2006). Trabecular bone, which makes up 20% of the skeletal mass and is found predominantly in the lumbar vertebra (LV) and proximal and distal ends of long bones, has a rate of remodeling that is approximately 5-10 times higher than the rate of remodeling of cortical bone in adults (Bell et al., 1967; Hadjidakis & Androulakis, 2006). Thus, trabecular bone is more metabolically active than cortical bone.
**Figure 2-1: Bone turnover (remodeling) in a bone multicellular unit (BMU).**

1. **Activation:** Local factors (e.g. receptor activator of nuclear factor kappa-B ligand (RANKL)) and systemic factors (e.g. increased parathyroid hormone, decreased estrogen) activate osteoclasts.
2. **Resorption:** Activated osteoclasts resorb bone to bore a hole through bone.
3. **Reversal:** Mononuclear cells assist in removing organic matrix remnants left by the osteoclasts and to assist in forming a cementum line at the resorption pit.
4. **Formation:** Formation of new bone is then initiated as osteoblasts fill the resorption pit with new matrix which then becomes mineralized.
5. **Quiescence:** Osteoblasts become osteocytes when embedded into the mineralized bone matrix or resting bone lining cells on the newly formed bone surface. Osteoblasts may also undergo apoptosis. Modified from (Raisz, 2005; Lerner, 2006).
Bone turnover takes place within temporary anatomical structures known as basic multicellular units (BMUs) located at the surface of specific sites of bone (e.g. periosteum, endosteum, haversian canals of cortical bone; trabeculae of trabecular bone) (Figure 2-1) and is tightly regulated by local and systemic factors such as cytokines and circulating hormones (Raisz, 2005; Lerner, 2006; Morgan et al., 2008). The remodeling cycle always proceeds through 5 phases: activation; bone resorption; reversal; bone formation; and quiescence (Figure 2-1). Since the activation phase is characterized by the activation of the bone-resorbing osteoclast, the remodeling cycle always begins with the osteoclast. Osteoclast activation by local and systemic factors initiates the resorption of bone by boring a hole through bone. Once the hole is formed, a brief reversal phase ensues whereby the bone surface is covered by mononuclear cells to assist in removing organic matrix remnants left by the osteoclasts and to assist in forming a cementum line. Formation of new bone is then initiated as osteoblasts fill the resorption pit with collagenous matrix which then becomes mineralized (Figure 2-1). The final phase, quiescence, proceeds whereby osteoblasts either terminally differentiate into osteocytes when embedded into the mineralized bone matrix or they become resting bone lining cells on the newly formed bone surface (Raisz, 2005; Lerner, 2006; Morgan et al., 2008).

Figure 2-2 illustrates in more detail than Figure 2-1, specific mediators that regulate interactions between cells of the osteoblast and osteoclast lineages, the dominant players of bone turnover. Activation of the remodeling cycle from local and systemic factors induces transcriptional responses in osteoblasts. These transcriptional responses include an increased expression of cytokines such as macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor κβ ligand (RANKL) and a decreased expression of the cytokine osteoprotegerin (OPG) (Lerner, 2006; Raggatt & Partridge, 2010) (Figure 2-2). M-CSF is necessary for osteoclast development from precursor cells of the hematopoietic cell lineage.
Figure 2-2: Graphic representation of the interactions between osteoblast lineage cells (A-C) and osteoclast lineage cells (D-F) which influence their differentiation and activation during the remodeling cycle.

Osteoblasts express the majority of cytokines that regulate osteoclast progenitor differentiation including macrophage colony-stimulating factor (M-CSF), receptor activator of nuclear factor κβ ligand (RANKL), and osteoprotegerin (OPG). M-CSF and RANKL bind to their receptors (c-fms and RANK, respectively) on the hematopoietic stem cell and pre-osteoclast which drives their differentiation and activation. OPG serves as a decoy receptor to balance the activity of RANKL. The ratio of OPG to RANKL determines the fate of the osteoclast and is a key regulator of bone resorption. Conversely, growth factors (e.g. TGF-β, BMP, etc) that are released from bone during osteoclast-mediated removal stimulate the expression of transcription factors Runx2 and osterix. Runx2 drives the commitment of mesenchymal stem cells to the osteoblast and chondrocyte lineages and, osterix drives their commitment exclusively to the osteoblast lineage. The coupling of numbers and activities of the mature osteoblast and osteoclast determines the balance between bone formation and bone resorption (bone turnover). Markers of bone resorption (tartrate resistant acid phosphatase-5β (TRAP-5β), C-terminal telopeptides of type I collagen (CTX), OPG, RANKL, OPG/RANKL), bone formation (osterix, osteocalcin) and overall bone turnover (osteocalcin) can be measured in the serum (TRAP-5β, CTX, osteocalcin, OPG, RANKL) or at the local tissue (TRAP-5β, osteocalcin, osterix, OPG, RANKL). Figure modified from (Morgan et al., 2008).
while RANKL is necessary for the differentiation, activation and survival of osteoclastic cells (Khosla, 2001). OPG serves as a decoy receptor of RANKL, which binds to RANK and inhibits RANKL-induced osteoclastogenesis, activation and survival. Thus, the OPG/RANKL ratio is the major determinant of osteoclast formation, activation and survival (Khosla, 2001; Leibbrandt & Penninger, 2008). The induction of transcriptional responses by the osteoblasts results in the recruitment of osteoclast precursors to the remodeling site and then results in their differentiation and activation. Osteoclasts in the BMU then go on to degrade the mineralized matrix in the resorption phase resulting in the release of a number of collagen fragment products (e.g. C-terminal telopeptides of type I collagen (CTX), N-terminal telopeptides of type I collagen (NTX), pyridinoline (PYD) and deoxypyridinoline (DPD)) and noncollagenous fragment products (e.g. osteocalcin, osteopontin) (Polak-Jonkisz, 1998). Growth factors, such as FGF and TGF-β are also released from the skeletal tissue which will later contribute to the recruitment and activation of osteoblasts to the resorbed bone (Lerner, 2006). Once the organic matrix remnants left by the osteoclasts are removed by mononuclear cells and a cementum line is formed at the bottom of the lacunae in the reversal phase, the formation phase ensues whereby osteoblast precursor cells from the mesenchymal lineage are recruited to the BMU where they differentiate into fully active osteoblasts (Raisz, 2005; Lerner, 2006; Morgan et al., 2008). Osteoblast differentiation occurs under the control of two master transcription factors, runt-related transcription factor 2 (Runx2) and osterix, which are stimulated by the growth factors (e.g. BMPs) that were released from bone during the resorption phase (Pereira et al., 2000; Lee et al., 2002a; Celil & Campbell, 2005; Shen et al., 2010). Runx2 drives the commitment of mesenchymal stem cells to the osteoblast and chondrocyte lineages, while osterix drives their commitment exclusively to the osteoblast lineage (Nakashima et al., 2002). The formation of new bone occurs with the production and secretion of type I collagen and specialized bone-
matrix proteins including osteocalcin, alkaline phosphatase, bone sialoprotein, osteopontin, and osteonectin from osteoblasts. These specialized proteins assist in matrix maturation and mineralization in the newly formed osteoid. In the quiescence phase, osteoblasts become either flat lining cells, osteocytes or they undergo apoptosis (Raisz, 2005; Lerner, 2006; Morgan et al., 2008).

2.1.1 Direct Effects of Estrogen on Bone Turnover

In adults, estrogen suppresses the activation frequency of BMUs (i.e. the number of new remodeling units activated in each unit of time) and bone resorption, resulting in the attenuation of bone turnover and subsequent bone loss (Komm et al., 2008; Khosla et al., 2011). Much of what we know about the action of estrogen on bone turnover is in relation to the estrogen receptor (ER)-mediated effects in the osteoblast, which subsequently influence the differentiation, activation and survival of osteoclasts.

ERs belong to the nuclear steroid hormone receptor gene superfamily and mediate most biological effects of estrogen. ER-alpha (ERα) and ER-beta (ERβ) are the two ER subtypes which contain five domains (A-F) with distinct functions to bind to ligands and DNA, to interact with coregulators and to affect the estrogen agonist/antagonist activities of compounds that can bind to the ER (ex: selective ER mediators) (Nilsson & Gustafsson, 2010; Nilsson & Gustafsson, 2011). Both ERs are present in skeletal tissue; cortical bone predominantly expresses ERα and contains little or no ERβ, while trabecular bone expresses both receptors with a greater abundance of ERβ (Onoe et al., 1997; Bord et al., 2001; Modder et al., 2004; Almeida et al., 2007). Both receptors play important roles for maintaining bone mass (Windahl et al., 2002; Sims et al., 2003) while in aging mouse models of sex hormone deficiency, ERα plays a greater role in the protective action of estrogen on bone (Sims et al., 2003).
Indeed, estrogen attenuates bone resorption by modulating transcriptional activity in osteoblasts and, to a lesser extent, by modulating transcriptional activity in osteoclasts (Michael et al., 2005). These transcriptional events induced by estrogen play a major role in bone cell metabolism by regulating the production, activity and survival of the bone-resorbing osteoclast (Komm et al., 2008). The major estrogen pathways that directly regulate bone turnover include: classical genomic ER pathway; non-classical genomic ER pathway and non-genomic ER signaling (Komm et al., 2008) (Figure 2-3). In the classical genomic ER pathway (Figure 2-3A), the binding of estrogen to ER in osteoblasts triggers conformational changes in the receptor that leads to dimerization and subsequent interaction of the liganded ER with estrogen response elements in the promoter region of estrogen-responsive genes (e.g. Fas-L). This interaction promotes the recruitment of coactivators that stimulate the transcription of genes (e.g. Fas-L) which exerts a pro-apoptotic effect in osteoclasts (Hofbauer et al., 2004; Nakamura et al., 2007; Komm et al., 2008). In the non-classical genomic ER pathway (Figure 2-3B), the liganded ER can indirectly regulate gene transcription through its recruitment to specific promoter complexes where it interacts with other DNA-bound transcription factors (e.g. Nuclear Factor kappa Beta [NF-kB]) leading to a decreased transcription of genes (e.g. M-CSF, interleukin-6 (IL-6) and TNF-α) that result in decreased production of RANKL and, ultimately decreased proliferation and increased apoptosis of osteoclasts (Manolagas & Jilka, 1995; Pacifici, 1996; Jilka, 1998; Suda et al., 1999) (Pro-inflammatory cytokines such as IL-6 and TNF-α stimulate osteoblasts to express RANKL). Through non-genomic signaling (Figure 2-3C), estrogen can activate rapid signaling pathways (MAPK-ERK pathway) downstream of growth factor receptors (e.g. IGF-1 and TGF-β). Activation of the MAPK pathway stimulates proliferation in osteoblasts and apoptosis in osteoclasts (Chen et al., 2005). It has been demonstrated that ERs located in the
Figure 2-3: Direct action of 17β-estradiol on osteoblast and osteoclasts cells. (A) the classical genomic estrogen receptor (ER) pathway is mainly defined in osteoblast cells. Binding of estrogen to the ER in the osteoblast stimulates the transcription of Fas-L which upon binding to its receptor on the osteoclast, induces osteoclast apoptosis, (B) the non-classical ER pathway is mainly defined in osteoblast cells. The liganded ER interacts with NF-κB leading to decreased transcription of genes that ultimately lead to a decreased production of RANKL and a subsequent increase in osteoclast apoptosis (C) the non-genomic signaling pathway is defined in both osteoblast and osteoclast cells. The liganded ER activates MAPK signaling to stimulate proliferation in osteoblasts and apoptosis in osteoclasts. Figure produced by S.Sacco (2010).
plasma membrane of osteoblasts and osteoclasts can induce rapid signaling pathways to also result in the stimulation of apoptosis in osteoclasts while simultaneously attenuating apoptosis in osteoblasts (Kousteni et al., 2001; Kousteni et al., 2003; Nakamura et al., 2007; Krum et al., 2008).

2.1.2 Indirect Effects of Estrogen on Bone Turnover

Estrogen induces a number of indirect effects on bone to maintain balanced bone remodeling and skeletal mass. Estrogen increases intestinal calcium absorption and decreases renal calcium excretion (Arjmandi et al., 1993; Ten Bolscher et al., 1999). There is also evidence that estrogen suppresses the production of reactive oxygen species, which have been shown to increase the transcription of TNF-α in osteoclasts (Jagger et al., 2005). Moreover, estrogen suppresses T cell proliferation and activation, which suppress the production of inflammatory and osteoclastogenic cytokines (TNF-α, interferon-γ) and, ultimately results in reduced bone turnover and greater protection to skeletal mass (Lorenzo et al., 2008; Pacifici, 2010).

2.1.3 Summary

Bone is dynamic tissue whereby continuous remodeling serves to maintain structural and metabolic homeostasis in the body. When the coupling action of the osteoclasts and osteoblasts are balanced, old bone is replaced by an equal amount of new bone thus, there is minimal net change in bone mass. Estrogen plays an important role in regulating bone turnover throughout the adulthood via direct mechanisms involving ER-mediated pathways, and via indirect mechanisms including the modulation of calcium absorption, production of reactive oxygen species and immunoregulation. Unbalanced remodeling occurs when one of the two processes (bone resorption, bone formation) are quantitatively unequal, often resulting in the loss of too much bone. This form of unbalanced bone remodeling is common in postmenopausal women
due to the depletion in endogenous estrogen production, resulting in an increase in bone turnover and an increased risk for the development of osteoporosis and fragility fractures.

2.2 Postmenopausal Osteoporosis

2.2.1 Descriptive Epidemiology and Risk Factors

Osteoporosis is a skeletal disease characterized by compromised bone strength, predisposing an individual to an increased risk of fractures (NIH Consensus Development Panel on Osteoporosis Prevention Diagnosis and Therapy, 2001) (Figure 2-4). Primary osteoporosis occurs in postmenopausal women between the ages of 50 and 70 (Type 1 osteoporosis) or in people over the age of 70 (Type 2 osteoporosis) largely because of a loss of estrogen during the menopause or the aging process, respectively, while secondary osteoporosis occurs in various populations, at various ages and is caused by a number of factors such as disease (e.g. hyperparathyroidism, hyperthyroidism, Crohns disease) and medication use (e.g. corticosteroids) (Templeton, 2005). The most common class of osteoporosis in postmenopausal women is primary osteoporosis due to the depletion in endogenous estrogen production.

Bone weakness results from both a loss in bone quantity and bone quality (e.g. structure) (Figure 2-4). According to the World Health Organization (WHO), the diagnosis of osteoporosis occurs when bone mineral density (BMD), as measured by dual energy x-ray absorptiometry (DEXA), is 2.5 standard deviations below the mean value for young sex-matched adults (WHO, 1994). However, because approximately half of all postmenopausal fractures occur in women with BMD scores that do not meet the diagnostic criteria of osteoporosis (Stone et al., 2003; Schuit et al., 2004), BMD is not always reliable marker in predicting fracture risk (Delmas & Seeman, 2004). Thus, determining other factors that contribute to bone strength, such as bone
Postmenopausal osteoporosis is a skeletal disease characterized by compromised bone strength, predisposing an individual to an increased risk of fractures (NIH Consensus Development Panel on Osteoporosis Prevention Diagnosis and Therapy, 2001). Bone weakness results from both a loss in bone quantity and bone quality. Assessment of bone mineral density (BMD) is commonly performed using dual-energy x-ray absorptiometry (DEXA) and provides a measure of the amount of mineral within a selected area of bone. In humans, BMD is a predictor of fracture risk and is routinely determined to diagnose osteoporosis. Assessment of bone structure provides a marker for bone quality and, is performed using imaging techniques such as peripheral quantitative computed tomography (pQCT) (in live humans) or micro-computed tomography (µCT) (in animal and human specimens). Computed tomography provides microarchitectural characteristics including trabecular number, trabecular separation, and trabecular thickness. Both the BMD and bone structure play a contributing role to bone strength and ultimately fracture risk. In animal models, bone strength can be directly determined by performing biomechanical strength testing of excised bones at specific skeletal sites.
quality (e.g. bone structure) can provide improved prediction of fracture risk (Roux et al., 2010; Stein et al., 2010).

The prevalence of osteoporosis in the United States and Canada is high, with 10 million Americans and 2 million Canadians affected with this disease (Harvey et al., 2010; Osteoporosis Canada, 2011). A number of notable risk factors contribute to the development of postmenopausal osteoporosis which includes both genetic and lifestyle factors (Table 2-1) (Nordin, 2008). Along with its significant consequences to morbidity and mortality, osteoporosis has an annual cost of 20 billion dollars to the American and Canadian health care systems combined and, it is expected that this economic burden will rise due to rapidly aging populations (Burge et al., 2007).

The most common sites of osteoporosis-related fragility fractures are at the wrist, vertebrae and hip. The consequences fragility fractures include chronic pain, decreased quality of life, loss of independence, fear of falling, development of restrictive lung disease, and increased mortality (Greendale et al., 1995; Ensrud et al., 2000; Adachi et al., 2001). Fractures may occur as a result of both a decrease in mineral content and matrix proteins at these sites. The consequences of low mineral and matrix proteins in lumbar vertebra are depicted in Figure 2-5, where bone mineral and bone matrix (Figure 2-5A) are replaced by spacious pores (Figure 2-5B). The result is a reduction in trabecular integrity, as the number and thickness of individual trabeculae are lower than the healthy bone and, the distance between each trabecula (trabecular separation) is greater compared to the healthy bone. The consequence is a weakened vertebra at high risk for compression fracture (Qiu et al., 2006; Wegrzyn et al., 2010).

2.2.2 Pathophysiology of Postmenopausal Osteoporosis

As discussed in Section 2.1.1, estrogen is a critical mediator of bone turnover. Thus, estrogen deficiency is a major contributor to bone loss, osteoporosis and risk of fragility fracture in
Table 2-1: Risk factors for osteoporosis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Direct Effect on Bone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of Estrogen</td>
<td>Positive</td>
</tr>
<tr>
<td>Presence of Androgen</td>
<td>Positive</td>
</tr>
<tr>
<td>Aging</td>
<td>Negative</td>
</tr>
<tr>
<td>Regular Exercise</td>
<td>Positive</td>
</tr>
<tr>
<td>Immobilization</td>
<td>Negative</td>
</tr>
<tr>
<td>Dietary Calcium</td>
<td>Positive</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>Positive</td>
</tr>
<tr>
<td>Excessive Alcohol Consumption</td>
<td>Negative</td>
</tr>
<tr>
<td>Smoking</td>
<td>Negative</td>
</tr>
<tr>
<td>Family History of Osteoporosis</td>
<td>Negative</td>
</tr>
</tbody>
</table>
Figure 2-5: Consequences of low bone mass and matrix proteins in the lumbar vertebra. Greater bone volume, higher trabecular number, trabecular thickness and lower trabecular separation are characteristic of a healthy vertebra (A), while lower bone volume, trabecular number, trabecular thickness and greater trabecular separation are characteristic of a vertebra with low bone mass and matrix proteins (B) which is typical in osteoporosis. Sacco, 2010 (unpublished work).
postmenopausal women. During the first 4-8 years after the onset of menopause, an accelerated phase of bone loss occurs whereby 20-30% of trabecular bone and 5-10% of cortical bone are lost (Riggs et al., 1998). A slower phase of bone loss then dominates resulting in a total net loss of approximately 20-30% of both trabecular and cortical bone (Riggs et al., 1998). Indeed, reduced levels of endogenous bioavailable estrogen in postmenopausal women are associated with lower BMD and higher risk for fragility fractures (Cummings et al., 1998; Bagur et al., 2004; Ongphiphadhanakul et al., 2004; Huang et al., 2007).

The decline in endogenous estrogen production that occurs during menopause results in an increased level of activation frequency of BMUs since, as described earlier, estrogen plays an important modulatory role on the expression of key cytokines (M-CSF, RANKL) that regulate bone turnover (since both the direct and indirect actions of estrogen include the suppression of pro-inflammatory cytokines such as IL-6 and TNF-α, the development of osteoporosis can be associated with an inflammatory component). However, within the BMU, the resorption phase dominates over the formation phase during menopause and results in a net loss of skeletal tissue (Lerner, 2006; Khosla et al., 2011). The reason for the enhanced resorption phase during estrogen depletion is mainly due to the prolonged lifespan of the osteoclast but it is also attributed by its increased activity. Figure 2-6 depicts the typical changes in gene expression that occur when endogenous estrogen levels are low. The increased expressions of M-CSF and RANKL drive osteoclast formation, survival and activity. The increase in osteoclast formation and activity can be depicted in postmenopausal women as an increase in circulating TRAP-5β levels (Peris et al., 1999; Qin et al., 2008) as well as in some (Miyazaki et al., 2004; Rahman et al., 2009) but not all (Ominsky et al., 2008; Park et al., 2008) studies using ovariectomized rats, a model reflective of the postmenopausal condition. This discrepancy within ovariectomized rats may be due to the timing since ovariectomy as well as method of analyses (Alatalo et al., 2003).
Figure 2-6: Effect of estrogen depletion on the interactions between osteoblast lineage cells (A-C) and osteoclast lineage cells (D-F).

A depletion in endogenous estrogen results in the increased transcription of cytokines (M-CSF, RANKL) by osteoblasts and stromal cells which stimulate the differentiation, activation and survival of osteoclast cells. This can be characterized by an increase in circulating CTX. Over time, the loss of bone results in a lower absolute number of osteoclasts since less bone is present and is characterized by lower TRAP-5β. Bone formation also increases in an attempt to “catch up” to the loss of bone that is occurring, which is characterized by increased osteocalcin levels (osteocalcin is also indicative of the rate of bone turnover). The remodeling cycle favors bone resorption for reasons which are currently unclear, resulting in a net loss of bone tissue. Figure modified from (Morgan et al., 2008).
However, the absolute number of osteoclasts in bone tissue declines after ovariectomy, which is depicted by lower TRAP-5β levels in the skeletal tissue (Alatalo et al., 2003). The decline in circulating estrogen also stimulates an increase in circulating CTX levels as a result in greater degradation of the bone matrix and hence, greater release of type I collagen products. While degradation of the matrix releases growth factors (e.g. TGF-β1, FGF) that are imbedded in bone, their effects on the expressions of Runx2 and osterix for osteoblast differentiation during situations of low endogenous estrogen levels are not clearly defined (Dalle Carbonare et al., 2009; Peng et al., 2010). Nevertheless, an increase in rate of bone formation occurs that is characterized by increased circulating levels of markers of osteoblast activity (e.g. osteocalcin), which does not “catch up” to the rate of bone resorption due to an augmentation in osteoblast apoptosis.

2.2.3 Summary

The integration of bone mass and bone structure are two key determinants of overall skeletal strength and, the depletion of endogenous estrogen that occurs during the menopause plays an important role on their loss and deterioration. A low level of circulating endogenous estrogen is mainly associated with an upregulation of cytokines that support the lifespan and activity of the osteoclast. While osteoblast activity also increases when circulating estrogen levels are low, the rate of bone formation by the osteoblasts cannot balance the rate of bone resorption by the osteoclasts, resulting in a net loss of skeletal tissue.

2.3 Estrogen Replacement Therapy for Postmenopausal Bone Loss

The repletion of endogenous estrogen through the use of hormone therapy (HT) effectively
prevents postmenopausal bone loss and reduces the risk of fragility fractures (The Writing Group for the PEPI Trial, 1996; Rossouw et al., 2002; Anderson et al., 2004; Pasco et al., 2004; Hundrup et al., 2005). The most popular HT formulation prescribed to postmenopausal women is CEE derived from urine of pregnant mares (Wysowski et al., 1995; Hersh et al., 2004; Davis et al., 2005). MPA is conventionally added to CEE for women with an intact uterus because estrogen induces endometrial hyperplasia and increases the risk for endometrial cancer (Grady et al., 1995). For hysterectomized women where endometrial cancer is not a concern, CEE alone is used for the treatment of postmenopausal-related conditions including loss of bone mass. CEE, as indicated by its name, is itself made up of several conjugated forms of estrogen, mainly estrone sulfate and equilin sulfate. The most prescribed dose of CEE is 0.625 mg/d because this is the dose that alleviates postmenopausal-related symptoms in the majority of women that endure ovarian failure (Gambacciani & Genazzani, 2001). Other forms of estrogen in HT include esterified estrogen, ethinyl estradiol, 17β-estradiol, estradiol acetate and estropipate. Norethindrone, levonorgestrel and progesterone are the commonly used forms of progestogens in HT.

2.3.1 Standard Dose Estrogen Replacement Therapy

2.3.1.1 Attenuation of Postmenopausal Bone Loss

Unquestionably, HT during the postmenopause improves BMD and decreases fracture risk in the vertebrae as well as in non-vertebral sites including the hip (Table 2-2) (Cauley et al., 1995; The Writing Group for the PEPI Trial, 1996; Rossouw et al., 2002; Anderson et al., 2004; Banks et al., 2004; Hundrup et al., 2005). Observational studies have reported decreases of up to 71% in fracture risk with HT use (Banks et al., 2004; Hundrup et al., 2005). In a subgroup (n= 138,737) of postmenopausal women from the Million Women Study, current use of HT was associated with a 38% decrease in risk for total fractures, regardless of the type, dose or delivery method of
Table 2-2: Selected studies on the effect of hormone therapy (HT) on bone metabolism in postmenopausal women

<table>
<thead>
<tr>
<th>Study Name, Study Type</th>
<th>Sample Size, Mean Age in Years (Range), Years since Menopause</th>
<th>Dose and Type of Therapy</th>
<th>Treatment Duration</th>
<th>Results for BMD, BMC or Fracture Risk</th>
<th>Reference</th>
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<tr>
<td>WHI Estrogen plus Progesterone Trial, RCT</td>
<td>16608, 63 (50-79), &gt;6 months (&gt;12 months for 50-54 year olds)</td>
<td>CEE: 0.625 mg/d + MPA: 2.5 mg/d (continuous), or placebo</td>
<td>Stopped after a mean of 5.2 years due to increases in breast cancer, stroke, CHD</td>
<td>•HR, 0.66 [0.45-0.98] for incidence of hip fractures •HR, 0.76 [0.69-0.85] for incidence of total fractures</td>
<td>(Rossouw et al., 2002)</td>
</tr>
<tr>
<td>WHI Estrogen alone Trial, RCT</td>
<td>10739, 64 (50-79), previously hysterectomized</td>
<td>CEE: 0.635 mg/d or placebo</td>
<td>Stopped after a mean of 6.8 years due to increases in stroke</td>
<td>•HR, 0.61 [0.41-0.91] for incidence of hip fractures •HR, 0.70 [0.63-0.79] for incidence of total fractures</td>
<td>(Anderson et al., 2004)</td>
</tr>
<tr>
<td>WISDOM Trial, RCT</td>
<td>4385, 63 (50-69), &gt;12 months or previously hysterectomized</td>
<td>CEE: 0.635 mg/d alone or + MPA: 2.5 mg/d (continuous) or or 5.0 mg/d (sequential), or placebo</td>
<td>Stopped after a mean of 11.9 months due to adverse findings in WHI</td>
<td>•HR, 0.69 [0.46-1.03] for incidence of osteoporotic fractures</td>
<td>(Vickers et al., 2007)</td>
</tr>
<tr>
<td>Study Name, Study Type</td>
<td>Sample Size, Mean Age in Years (Range), Years since Menopause</td>
<td>Dose and Type of Therapy</td>
<td>Treatment Duration</td>
<td>Results for BMD, BMC or Fracture Risk</td>
<td>Reference</td>
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<tr>
<td>PEPI Trial, RCT</td>
<td>875, 56 (45-64), 1 to &lt;10 years (either naturally or surgically)</td>
<td>CEE: 0.625 mg/d alone or + either MPA: 2.5 mg/d (continuous) or 10 mg/d (sequential), or MP: 200 mg/d (sequential), or placebo</td>
<td>3 years</td>
<td>•3.5-5.0% increase in LV BMD •1.7% increase in hip BMD</td>
<td>(The Writing Group for the 1996)</td>
</tr>
<tr>
<td>Danish Nurse Cohort Study, Prospective</td>
<td>14015, 60 (≥ 50), Years since menopause not provided</td>
<td>All doses and types of therapy (e.g. CEE, &gt;0.625 mg/d or ≤0.625 mg/d; E2, &gt;1 mg or ≤1 mg oral; E2, &gt;50 µg or ≤50 µg transdermal; Various doses of MPA and NA as continuous or sequential regimes)</td>
<td>Mean years of follow-up: Not reported (Range: 0-6 years)</td>
<td>•HR, 0.69 [0.50-0.94] for incidence of hip hip fractures with ever users</td>
<td>(Hundrup et al., 2005)</td>
</tr>
<tr>
<td>Million Women Study, Prospective</td>
<td>138737, Mean age in years not provided (50-69), Years since menopause not provided</td>
<td>All doses and types of therapy (e.g. CEE, &gt;0.625 mg/d or ≤0.625 mg/d; E2, &gt;1 mg or ≤1 mg oral; E2, &gt;50 µg or ≤50 µg transdermal; Various doses of MPA and NA as continuous or sequential regimes)</td>
<td>Mean years of follow-up: 2.8 (Range: 1.9-3.9 years)</td>
<td>•RR, 0.62 [0.58-0.66] for incidence of fracture with current users •RR, 1.07 [0.99-1.15] for incidence of fracture with past users</td>
<td>(Banks et al., 2004)</td>
</tr>
<tr>
<td>Study Name, Study Type</td>
<td>Sample Size, Mean Age in Years (Range), Years since Menopause</td>
<td>Dose and Type of Therapy</td>
<td>Treatment Duration</td>
<td>Results for BMD, BMC or Fracture Risk</td>
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| SOF Trial, Prospective | 9704, 70-72 (>65), 2 years before onset of menopause, to > 10 years | All doses and types oral preparations | Mean years of follow-up: 2.5 (Range: 0.02-6.5 years) | •RR, 0.60 [0.36-1.02] for incidence of hip fractures with current users  
•RR, 0.66 [0.54-0.80] for incidence of all nonspinal fractures with current users  
•RR, 0.29 [0.09-0.92] for incidence of hip fractures with current users who started HT within 5 years of menopause  
•RR, 0.50 [0.36-0.70] for incidence of all nonspinal fractures with current users who started HT within 5 years of menopause | (Cauley et al., 1995) |

BMC= bone mineral content, BMD= bone mineral density, CEE= conjugated equine estrogen, $E_2$= 17β-estradiol, HR= hazard ratio, LV= lumbar vertebra, MP= micronized progesterone, MPA= medroxyprogesterone acetate, NA= norethisterone acetate, PEPI= postmenopausal estrogen/progestin interventions trial, RCT= randomized control trial, RR= relative risk, SOF= study of osteoporotic fractures, WHI= women’s health initiative trial, WISDOM= women's international study of long duration oestrogen after menopause.
HT (Banks et al., 2004). Similar observations were reported in other prospective cohorts including the Study of Osteoporotic Fractures trial, whereby current use of HT was associated with 34% and 38% decreases in risk for all nonspinal and hip fractures, respectively (Cauley et al., 1995). Moreover, current users that started HT within 5 years of menopause had a 50% decrease in risk for all nonspinal fractures and a 71% decrease in risk of hip fractures (Cauley et al., 1995).

Similar to observational studies, randomized control trials (RCT) have confirmed the protection of HT against fracture risk, albeit at more modest levels (~30%) (Table 2-2) (Rossouw et al., 2002; Anderson et al., 2004; Vickers et al., 2007). The largest RCT reporting the effects of HT on fracture risk is the WHI. This trial was designed primarily to determine whether daily use of 0.625 mg CEE plus 2.5 mg MPA (for non-hysterectomized women) or 0.625 mg CEE alone (for hysterectomized women) reduces coronary heart disease (CHD) in healthy postmenopausal women (Rossouw et al., 2002; Anderson et al., 2004). Both the combination (CEE + MPA) and alone (CEE) arms were stopped early, after a mean of 5.2 and 6.8 years, respectively, because the number of observed side effects was deemed unacceptable. Nevertheless, CEE + MPA use was associated with a 34% decrease in risk for hip fractures, and a 26% decrease in risk for total fractures (Rossouw et al., 2002). Use of CEE alone was associated with a 39% decrease in risk for hip fractures, and a 30% decrease in risk for total fractures (Table 2-2) (Anderson et al., 2004).

2.3.1.2 Adverse Effects

While the benefit of HT, particularly CEE, on the prevention and treatment of postmenopausal osteoporosis and related fracture has been demonstrated, its effects on the health of other estrogen-sensitive tissues must be carefully considered. In many circumstances, estrogen induces proliferation of the uterine and breast tissues (Punyadeera et al., 2003; Cordera &
Jordan, 2006; Lewis-Wambi & Jordan, 2009). In addition, HT has the ability to modulate the health of the liver, kidneys and cardiovascular tissues since ERs are present in these tissues as well (Gustafsson, 1999). Since postmenopausal women are at a higher risk for chronic diseases such as CHD and breast cancer compared to their premenopausal counterparts, the effects of HT may modify these risks.

Previously described as an estrogen-deficiency disease, menopause was portrayed as a catalyst for the development of other disease such as CHD, due to the dramatic decline in endogenous estrogen production (Wilson, 1966; Davis et al., 2005). Observational trials indicated HT as a postmenopausal therapy with many health benefits and HT was marketed to prolong youth and libido, resulting in its popularity to quickly expand worldwide however, its effect on cardiovascular health has become rather controversial (Davis et al., 2005). While evidence from observational trials (Stampfer et al., 1991; Grodstein et al., 2000) suggest that HT is associated with a lower risk of CHD, subsequent data from two large RCTs, the Heart and Estrogen/Progestin Replacement Study (HERS) and the WHI trial, did not confirm these benefits (Hulley et al., 1998; Rossouw et al., 2002; Anderson et al., 2004). The HERS and WHI trials were originally designed to validate the view that HT supports health during menopause and prevents the development of side-effects associated with other diseases (e.g. CHD). The HERS and WHI trials were carried out on a combined 30110 postmenopausal women in multi-centre, randomized, double-blind, placebo-controlled designs (Hulley et al., 1998; Rossouw et al., 2002; Anderson et al., 2004). The HERS trial was the first large RCT to evaluate whether daily use of 0.625 mg CEE plus 2.5 mg MPA alters the risk of CHD events in 2763 postmenopausal women with established CHD (Hulley et al., 1998). After an average follow-up of 4.1 years, HT did not confer protection against cardiovascular events (non-fatal myocardial infarction or CHD death) and its use was associated with higher venous thromboembolic events compared to placebo.
Interestingly, HT use was associated with higher risk for CHD events during the first year of treatment however, in the fourth and fifth years of the trial, its use was associated with lower risk for CHD events compared to placebo. Thus, the HERS trial demonstrated that the use of HT does not provide an overall benefit to cardiovascular health in women that have never used HT but it may provide benefit to cardiovascular health in current users that continue HT (Hulley et al., 1998). Subsequently, the WHI, which was the largest RCT to examine the effect of HT on the prevention of CHD, demonstrated that HT is associated with increased risk of CHD, stroke, venous thromboembolism and breast cancer in the combination group and, stroke in the estrogen alone group. The WHI findings resulted in early termination of the trial and subsequently widespread discontinuation of HT among postmenopausal women worldwide (Rossouw et al., 2002; Anderson et al., 2004; Hersh et al., 2004; Ness et al., 2005).

The reasons for inconsistent findings among previous observational trials and the HERS and WHI trials may be due to differences in subject characteristics and inherent designs. For example, observational studies that have investigated the safety of HT have included younger postmenopausal subjects however, the HERS and WHI trials consisted of mainly older postmenopausal women. In addition, observational studies can be subjected to unknown confounders that influence the outcome variable and are susceptible to a number of biases including sampling bias and admission rate bias, whereas RCTs can experience inadequate randomization, unblinding, and loss of sampling units- all of which can influence the outcome variable (Heaney, 2008). Nevertheless, since RCTs by design are superior compared to observational studies, the WHI study would inevitably have the greatest impact on HT recommendations for the postmenopausal population.
2.3.1.3 Limitations of the Women’s Health Initiative Trial

Important limitations of the WHI have become the focus of subsequent dialogue and have stimulated experts to question whether the WHI findings can be generalized to all postmenopausal women receiving various forms of HT. These limitations include: i) a high loss of subjects; ii) an older and healthy postmenopausal population included as subjects; and iii) the investigation of one formulation, dose and delivery method of HT.

i) A high loss of subjects: The WHI suffered from a large loss of subjects. 42% of the subjects in the combination arm and 53.8% in the estrogen alone arm dropped out of the WHI resulting in reduced power of its design. The implication of a high drop-out rate may be an underestimation of observed effects, whether negative (e.g. CHD and breast cancer) or positive (e.g. fracture) (Rossouw et al., 2002). However, being that losses in subjects are rarely a random occurrence (Heaney, 2008), it is too simplistic to assume that the observed effects are merely an underestimation of what would have occurred if an appropriate number of subjects remained in the study throughout its total duration.

ii) Older, healthy postmenopausal women: The WHI studied mostly older and healthy postmenopausal women. While the WHI reported an unfavorable risk to benefit ratio, this study was conducted in a predominantly older and asymptomatic postmenopausal population, many of which who were 13-15 years past the onset of menopause. However, HT is targeted primarily to younger postmenopausal women for the treatment of menopausal symptoms including hot flushes, vaginal dryness and decreased libido. Thus, there is a clear disconnect between the conventional target group of HT and the subjects from the WHI in terms of age and health status. The question of whether similar findings would be observed in a younger postmenopausal population remains.
Observational studies suggest that time since menopause may be an important factor on risk of CHD and other side effects of HT (Pines et al., 2008; Hodis & Mack, 2009). Post-hoc subgroup analysis by Roussouw et al. (Rossouw et al., 2007) of younger postmenopausal women (aged 50-59) in the WHI trials detected a trend for HT to reduce the risk of CHD and total mortality. Further analysis of the WHI (Manson et al., 2007) showed that CEE alone resulted in lower coronary-artery calcification compared to placebo in the younger postmenopausal women aged 50-59 years. Nonetheless, these subsequent analyses were not adequately powered, especially in women aged 50-54 or closer to the onset of menopause (<5 years) (Manson et al., 2007). Thus, difficulties remain in drawing clear conclusions from the secondary analyses of the WHI. While data from observational trials have shown that HT is protective against CHD, mortality and other diseases in younger postmenopausal women, RCTs are underway to determine whether these previous findings can indeed be confirmed (Harman et al., 2005). In March 2008, the International Menopause Society held a global summit to address the actual versus perceived risks of HT use in younger postmenopausal women (Pines et al., 2008). Through the scrutiny of vast empirical evidence, this summit resulted in a consensus that there may be a “window of opportunity” whereby HT is safe in women that are within 10 years since the onset of menopause. Nevertheless, current opinion regarding the use of HT for the treatment of vasomotor symptoms and for the prevention of osteoporosis during the postmenopause is that the lowest effective dose should be used for the shortest duration possible.

iii) Investigation of one formulation, dose and delivery method of HT: Only two formulations of HT were investigated in the WHI, CEE + MPA (0.625 mg/d + 2.5 mg/d) for postmenopausal women with an intact uterus or CEE alone (0.625 mg/d) for postmenopausal women who had undergone hysterectomy. Both these formulations were provided as an oral tablet. Because more side effects were observed with the CEE+MPA formulation compared to
the CEE alone formulation, it is of question whether adverse effects may be in part due to MPA rather than CEE. Whether alternative formulations of HT, such as low-dose transdermal HT (LD), result in fewer side effects than those observed with the HT formulations used in the HERS or WHI trials are yet to be determined. However, emerging data suggest an advantage for the use of lower doses of HT through the transdermal delivery method compared to oral standard dose HT.

2.3.2 Lower Doses of Estrogen Replacement Therapy

The most commonly prescribed HT for postmenopausal women are oral CEE (0.625 mg/d) alone or in combination with MPA (2.5 mg/d). To determine the lowest-effective dose for the attenuation of menopausal-related conditions including bone loss, several studies chose to examine doses that are three-quarters, half, and a quarter of the standard dose (Table 2-3). In addition, alternative modes of delivery, such as the transdermal patch, are being studied because they are associated with fewer side effects compared to the oral standard dose formulations. Moreover, lower doses of transdermal estrogen monotherapy can potentially be used in women with an intact uterus without concomitant use of progesterone since lower doses of estrogen monotherapy have been shown to not stimulate hyperplasia of endometrial tissue (Ettinger et al., 2004; Richman et al., 2006; Ettinger, 2007; Samsioe et al., 2007). For the purpose of this thesis, 0.3 mg and 0.45 mg CEE, along with 25 µg transdermal 17β-estradiol are considered LD, while 14 µg transdermal 17β-estradiol is considered ULD (Table 2-3).

2.3.2.1 Attenuation of Postmenopausal Bone Loss

Results from a number of trials have shown that lower doses of HT are effective in improving BMD in the hip and lumbar spine (Table 2-4) (Recker et al., 1999; Gambacciani et al., 2001; Lindsay et al., 2002; Notelovitz et al., 2002; Prestwood et al., 2003; Ettinger et al., 2004; Garcia-Perez et al., 2006; Gambacciani et al., 2008; Schaefers et al., 2009). In a substudy (n=822) of the
Table 2-3: Examples of common doses and routes of administration of estrogen in hormone therapies.

<table>
<thead>
<tr>
<th>Route of Administration</th>
<th>Formulation</th>
<th>Standard Dose</th>
<th>Low-Dose</th>
<th>Ultra-Low-Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oral</td>
<td>CEE</td>
<td>0.625 mg/d</td>
<td>0.45 or 0.3 mg/d</td>
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<tr>
<td>Transdermal</td>
<td>17β-estradiol</td>
<td>0.05 mg/d</td>
<td>0.025 mg/d</td>
<td>0.014 mg/d</td>
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</table>

CEE= conjugated equine estrogen
<table>
<thead>
<tr>
<th>Study Name, Study Type</th>
<th>Sample Size, Mean Age in Years (Range), Years since Menopause</th>
<th>Dose and Type of Therapy</th>
<th>Treatment Duration</th>
<th>Results for BMD, BMC or Fracture Risk</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ULTRA Trial, RCT</td>
<td>417, 67 (60-80), ≥5 years</td>
<td>E₂ patch: 0.014 mg/day or placebo patch*</td>
<td>2 years</td>
<td>•2.1% greater LV BMD vs. placebo</td>
<td>(Ettinger et al., 2004)</td>
</tr>
<tr>
<td>HOPE Trial, RCT</td>
<td>822, 51.6 (40-65), 1 to ≤4 years</td>
<td>CEE: 0.625, 0.45 or 0.3 mg/d MPA: 2.5 or 1.5 mg/d or placebo*</td>
<td>2 years</td>
<td>•1.33-3.46% increase in LV BMD •1.5-3% increase in hip BMD •1.03-1.74% increase in total BMC</td>
<td>(Lindsay et al., 2002)</td>
</tr>
<tr>
<td>Gambacciani et al, 2008, Open Trial</td>
<td>Sample size not provided, 57 (range not provided), ≥1 year</td>
<td>1 mg E₂ + 0.5 mg NA for 28 d or 0.5 mg E₂ + 0.5 mg E₂ + 0.25 mg NA per day (oral), or no treatment*</td>
<td>2 years</td>
<td>•2-5% increase in LV BMD •1.8-2.8% increase in femur neck BMD</td>
<td>(Gambacciani et al., 2008)</td>
</tr>
<tr>
<td>Garcia-Perez et al, 2006, Transversal Study</td>
<td>136, 53 for 0.05 mg/d and placebo groups to 56 for 0.025 mg/d group, (range not provided), ≥1 year</td>
<td>E₂ patch: 0.05 or 0.025 mg/d + micronized progesterone: 100 mg/d, or placebo*</td>
<td>18 months</td>
<td>•0.73-0.92% increase in femur neck BMD •-0.35-0.87% change in LV BMD</td>
<td>(Garcia-Perez et al., 2006)</td>
</tr>
<tr>
<td>Gambacciani et al, 2001, Open Trial</td>
<td>38, 54 (45-56), ≥1 year</td>
<td>CEE: 0.3 mg/d MPA: 2.5 mg/d*</td>
<td>2 years</td>
<td>•2.7% increase in LV BMD</td>
<td>(Gambacciani et al., 2001)</td>
</tr>
</tbody>
</table>

BMC= bone mineral content, BMD= bone mineral density, CEE= conjugated equine estrogen, E₂= 17β-estradiol, HOPE= heart outcomes prevention evaluation, LV= lumbar vertebrae, MPA= medroxyprogesterone acetate, NA= norethisterone acetate, RCT= randomized control trial, ULTRA= ultra-low-dose transdermal estrogen assessment.

*All subjects received additional calcium alone or with vitamin D.
Women’s Health, Osteoporosis, Progestin, Estrogen (HOPE) trial, standard and lower-doses of orally administered CEE alone or in combination with MPA resulted in significant improvements from baseline in spine and hip BMD, as well as biochemical markers of bone turnover in healthy postmenopausal women who were within 4 years of the onset of menopause (Lindsay et al., 2002). These improvements contrasted from the placebo group which experienced significant losses in spine BMD and total body bone mineral content (BMC). While CEE alone at the standard dose resulted in higher gains in spine BMD compared to CEE alone at the 0.3 mg/d dose, the observed gains in BMD did not differ from the CEE alone at the 0.45 mg/d dose. In addition, no differences in gains in hip BMD were observed between any of the treatment groups. Significant benefits in spine BMD of up to 5% have also been reported with the use of LD in older postmenopausal women with low bone mass (Recker et al., 1999; Gambacciani et al., 2001; Lindsay et al., 2002).

The search for the lowest effective dose of HT has also been investigated using the transdermal delivery method (Notelovitz et al., 2002; Garcia-Perez et al., 2006). In healthy postmenopausal women, both standard- and LD transdermal 17β-estradiol with 100 mg/d micronized progesterone (MP) resulted in similar significant increases in femur neck BMD after 18 months of treatment, while the control group experienced a significant decrease in BMD (Garcia-Perez et al., 2006) (Table 2-4). Another study observed significant improvements in spine BMD in healthy hysterectomized postmenopausal women that were treated for 2 years with unopposed standard- and LD transdermal 17β-estradiol, respectively (Notelovitz et al., 2002). Testing the effectiveness of unopposed ULD transdermal 17β-estradiol, the Ultra-Low-dose Transdermal estRogen Assessment (ULTRA) observed a significantly greater increase in BMD at the lumbar spine in non-hysterectomized postmenopausal women after 2 years of treatment compared to placebo (Ettinger et al., 2004). A non-hysterectomized sample was used
since endometrial safety was also examined. Improvements in hip BMD and favorable changes in biochemical markers of bone turnover (osteocalcin and bone-specific alkaline phosphatase) were also observed from ULD (Ettinger et al., 2004).

While no large head-to-head studies have been conducted between lower doses of HT and other pharmacologic agents used for the prevention or treatment of postmenopausal bone loss, ULD via the transdermal delivery has also been shown to exert similar protection against bone loss in the spine but not at the hip as raloxifene, a selective ER modulator (Schaefers et al., 2009). In a 2-year RCT conducted in 500 osteopenic postmenopausal women, 77.3% of those treated with ULD and 80.5% of those treated with raloxifene groups did not experience bone loss at the lumbar spine. Slight increases in total hip BMD were observed in both the ULD and raloxifene group and, raloxifene resulted in significantly higher hip BMD compared to ULD. In addition, 63.8% of the ULD and 81.3% of the raloxifene group did not experience a loss of BMD at the hip. No difference in fracture incidence was observed between groups but trials that are longer in duration are needed to adequately determine whether lower doses and alternative methods of delivery of HT modulate fracture risk (Schaefers et al., 2009).

2.3.2.2 Adverse Effects

Studies have shown that the dose and formulation of HT can be significant factors in determining the safety profile of HT. In the transdermal delivery system, 17β-estradiol is absorbed subcutaneously and is readily absorbed into tissues as it circulates systemically before reaching the liver. Because transdermal HT avoids first-pass liver metabolism, there is lower production of coagulation factors (e.g. factor VII activity) compared to oral formulations which may result in a lower risk for cardiovascular events (Stevenson et al., 2004; Stevenson, 2009). Thus, lower doses of transdermal HT have the potential to provide significant benefit to skeletal health, but
with fewer side effects than the conventionally prescribed standard dose HT (Peeyananjarassri & Baber, 2005).

Evaluation of adverse events was included in the HOPE trial whereby a dose-response in endometrial hyperplasia and vaginal bleeding was observed in women taking CEE alone, with the greatest number of reported adverse effects with the 0.625 mg/d dose (Lindsay et al., 2002). The ULTRA trial, which examined the effects of unopposed transdermal ULD (17β-estradiol) on BMD and biochemical markers of bone turnover in non-hysterectomized postmenopausal women did not observe higher incidences of endometrial hyperplasia compared to the placebo group (Ettinger et al., 2004). In addition, further examination of the ULTRA trial by Grady et al (Grady et al., 2007) demonstrated that unopposed transdermal ULD (17β-estradiol) does not significantly change breast density in postmenopausal women. Another study showed that similar to raloxifene, no significant change in breast density in osteopenic postmenopausal women with 2 years treatment with unopposed transdermal ULD (17β-estradiol) (Nielsen et al., 2009). Whether reduced breast density from ULD estrogen therapy ultimately results in reduced incidence of breast cancer is not yet known. Indeed, there is a necessity for long-term trials to assess whether lower doses of HT using alternative modes of delivery affect disease risk profiles.

A lower risk profile observed with the use of lower-doses of estrogen therapy, in particular, transdermal estrogen monotherapy, makes HT an attractive option for younger postmenopausal women as they are indeed the target population of this therapy. Since lower-doses of estrogen monotherapy are being investigated in women with an intact uterus, its safety profile on the endometrium is a particular focus.

### 2.3.3 Summary

The benefit of HT for the prevention and treatment of postmenopausal bone loss is well acknowledged. However, due to its well-known ability to modulate physiology and subsequent
disease risk in other tissues, the safety of HT must always be considered before it is prescribed. Standard dose HT through the oral delivery method is effective against both postmenopausal bone loss and vasomotor symptoms however, inconsistencies in its safety have been noted between observational and RCTs. While review of the studies to date reveals discrepancies among studies and limitations of trials using standard doses of HT, emerging data suggests that HT can be safe for younger postmenopausal women. Moreover, lower doses of HT and alternative modes of delivery such as the transdermal patch may be a safer alternative to standard HT in protecting against postmenopausal bone loss while relieving vasomotor symptoms. The finding that LD transdermal estrogen therapy does not induce endometrial hyperplasia means that women who are not hysterectomized can benefit from estrogen monotherapy. However, further investigation in studies of longer duration (>2 years) are needed to evaluate the long-term effects of lower doses of transdermal estrogen therapy. These investigations can have important implications for women that use HT since combination therapy using the standard CEE+MPA formulation for nonhysterectomized women is associated with more side effects than the standard CEE alone. Another consideration is that many women who take pharmacologic agents such as HT are more likely to consume dietary supplements, many of which contain phytoestrogens (Mahady et al., 2003; Singh & Levine, 2006). Thus, determining the effects of these natural therapies, alone or combined with HT can help to establish an effective and safer treatment for postmenopausal bone loss. Flaxseed (FS) is an example of a commonly consumed food rich in phytoestrogens.
2.4 Flaxseed and Its Components

2.4.1 Flaxseed

FS (*Linum usitatissimum*) is an oilseed composed of approximately 29% carbohydrates, 18% protein, and 42% fat (USDA Nutrient Database, 2009). FS is rich in fiber and also contains appreciable levels of plant sterols however, it contains exceptionally high levels of lignans and the n-3 PUFA, ALA. FS and its components have become increasingly available in the Western diet (Fitzpatrick, 2007), which may be in part due to their potential to modulate the development or progression of a number of diseases such as breast cancer, cardiovascular disease, and diabetes (Lucas *et al.*, 2002; Dodin *et al.*, 2005; Thompson *et al.*, 2005; Dupasquier *et al.*, 2007; Pan *et al.*, 2007; Lindahl *et al.*, 2011).

2.4.1.1 Flaxseed Lignans

Lignans along with the isoflavones and coumestans are the major classes of phytoestrogens, plant compounds that can exert weak estrogenic and antiestrogenic action in the body. FS contains exceptionally high amounts of lignans and is the richest plant source of the lignan SDG (Thompson, 2003). The concentration of lignans from FS is approximately 0.165%-0.375% depending on the differences in species and samples as well as methods of analysis (Milder *et al.*, 2005; Penalvo *et al.*, 2005; Thompson *et al.*, 2006; Smeds *et al.*, 2007; Peterson *et al.*, 2010). Moreover, the SDG in FS is 75-800 times higher than the amount of SDG in other oilseeds, cereals, fruits, vegetables and legumes (Thompson *et al.*, 1991). While SDG is the principal lignan found in FS, other lignans such as matairesinol, pinoresinol, lariociresinol are also present in appreciable amounts (Thompson *et al.*, 1991; Chen *et al.*, 2006).

FS lignans are present in the defatted fractions of the seed. Upon ingestion of FS, SDG undergoes deglycosylation, demethylation, and dehydroxylation by the action of intestinal and colonic microflora to produce the mammalian lignan ED. ED can then undergo oxidation to
produce another mammalian lignan, EL (Figure 2-7) (Axelson *et al.*, 1982; Borriello *et al.*, 1985; Wang *et al.*, 2000). Other lignans (matairesinol, lariciresinol, pinoresinol) present in FS in smaller quantities are also metabolized to the mammalian lignans. ED and EL are then absorbed through the colonic barrier and are largely conjugated into glucuronides or sulfates or eliminated through the colon. The conjugated and free mammalian lignans that circulate systemically get excreted through urine, undergo enterohepatic circulation or in the case of free ED and EL, may also enter target tissues (Thompson, 2003).

ED and EL share structural similarity to endogenous 17β-estradiol (Figure 2-7). EL can bind to the ER with preferential binding to the ER-α (Mueller *et al.*, 2004; Penttinen *et al.*, 2007). Thus, the mammalian lignans can exert estrogenic and antiestrogenic effects in the body. For example, a physiological level of EL injected in ovariectomized mice has been shown to activate ER-mediated transcription in vitro and to stimulate uterus stromal edema and markers of proliferation (cyclin D1, Ki-67) in the uterus (Penttinen *et al.*, 2007) demonstrating the estrogenic ability of the mammalian lignan, EL. On the other hand, EL has been shown to reduce the relative uterine weight of adult intact female rats (Saarinen *et al.*, 2002) and reduce the growth of estrogen-responsive breast tumors under high circulating levels of estrogen (Bergman Jungestrom *et al.*, 2007). Thus, the mammalian lignans can exert estrogenic or antiestrogenic effects depending on the endogenous levels of estrogen.

The mammalian lignans also have the potential to alter the metabolism, bioavailability and action of estrogen. For example, in healthy postmenopausal women, 16 weeks of FS feeding (25 g/day) has shown to increase urinary concentrations of the non-estrogenic 17β-estradiol metabolite 2-hydroxyestrone, but not the estrogenic 17β-estradiol metabolite 16α-hydroxyestrone, and has shown that their ratio is positively correlated with urinary lignan excretion suggesting that FS modulates estrogen metabolism to favor a less estrogenic
Figure 2-7: Metabolic conversion of secoisolariciresinol diglycoside (SDG) to mammalian lignans, enterodiol (ED) and enterolactone (EL).

The distance between the hydroxyl appendages on the benzene rings of the mammalian lignans are similar to that on the 17β-estradiol structure, which allows the mammalian lignans to bind to the estrogen receptor and induce weak estrogenic or antiestrogenic effects on hormone sensitive tissues.
environment potentially in part due to its lignans (Brooks et al., 2004). In contrast, others have demonstrated that 12 weeks of FS feeding (7.5 g/day for 6 weeks followed by 15 g/day for 6 weeks) can increase the urinary levels of 16α-hydroxyestrone (Sturgeon et al., 2010) in healthy postmenopausal women suggesting that lignan-rich FS may induce an estrogenic response in the body. Thus, the action of lignans and FS can depend on a number of factors such as duration, timing and dose of treatment as well as concentration of endogenous estrogen. Thus, due to their structural similarity to 17β-estradiol and their ability to modulate 17β-estradiol action in the body, there is biological plausibility for the lignans from FS to modulate hormone-related diseases such as postmenopausal osteoporosis.

2.4.1.2 Flaxseed Oil

The fat content of FS is approximately 42% consisting of predominantly ALA (45-60%) and smaller amounts of oleic, linoleic, palmitic and stearic acids (Cunnane, 2003; Daun et al., 2003; USDA, 2009). The ALA content in FS is comparable to that in perilla seed and some edible plants (purslane, turnip, mushroom) (Cunnane, 2003) however, its wide availability and versatility in the food supply makes FS a convenient food to consume and, an attractive food to study the effect of omega-3 fatty acids in health and disease.

ALA is an n-3 PUFA which cannot be synthesized by the body due to a lack of the Δ15 desaturase enzyme but is essential for survival. Consequently, ALA is defined as an essential fatty acid (EFA) and it must be obtained from the diet. Upon ingestion and absorption, ALA is mainly beta-oxidized as fuel in various tissues while smaller amounts are accumulated into tissues, used for lipid synthesis or are excreted (Cunnane, 2003). ALA can also be converted to longer chain fatty acids including EPA and DHA through the action of the delta-6 and delta-5 desaturase enzymes (Figure 2-8). EPA can then form the precursor of the 3 series
n-3 and n-6 polyunsaturated fatty acids (PUFA) compete for metabolism into longer chain fatty acids because they share the same series of enzymes for metabolism. α-linolenic acid (ALA) is metabolized to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) while linoleic acid (LA) is metabolized to arachidonic acid (AA). The products of EPA and DHA are associated with anti-inflammatory action while the products of AA are associated with pro-inflammatory action in the body.

Figure 2-8: Metabolic conversion of n-3 and n-6 fatty acids.
prostaglandins, 5 series leukotrienes and E series resolvins. DHA can form the precursor of the D series resolvins and protectins.

Conversion from ALA to EPA is estimated to be between 0.2-6%, while its conversion to DHA is estimated to be 0.05% or less. However, a gender-related difference exists whereby conversion of ALA to EPA and DHA is considerably greater (2.5 times greater for EPA, >200 times greater for DHA) in women than in men (Burdge & Wootton, 2002). This gender-related difference in ALA metabolism is hypothesized to be related to endogenous estrogen, as HT use has also been shown to increase plasma EPA and DHA in postmenopausal women (Sumino *et al.*, 2003; Giltay *et al.*, 2004).

The bioactivity of ALA in the body is believed to be mainly due to its ability to decrease n-6 PUFA production of pro-inflammatory compounds such as PGE₂ and the 4 series leukotrienes (Figure 2-8) (Calder, 2006). More specifically, ALA and its longer-chain derivatives, EPA and DHA, can decrease n-6 PUFA production of pro-inflammatory compounds as a result of their competition for the same series of enzymes that are shared for n-3 and n-6 PUFA conversion (Figure 2-8). In addition, EPA and DHA produce anti-inflammatory eicosanoids such as the 3 series prostaglandins, resolvins and protectins (Calder, 2009). Thus, when EPA or DHA are cleaved from the cell membrane by phospholipase A₂, their metabolism into anti-inflammatory eicosanoids and eicosanoids metabolites result in the suppression of NF-κB activation and subsequent production of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (Schmitz & Ecker, 2008). This contrasts the effects of when arachidonic acid is cleaved from the cell membrane by phospholipase A₂, its metabolism into pro-inflammatory eicosanoids results in the production of pro-inflammatory cytokines including TNF-α, IL-1 and IL-6. Other mechanisms whereby the metabolites of ALA exert bioactivity include their ability to alter membrane fluidity
and phospholipid composition which can alter protein function and trafficking within the cell to modulate gene expression and cell survival (Nakamura et al., 2001). At present, it is unclear whether the bioactivities of the n-3 PUFA can be attributed to a direct effect from ALA or, if they are the result of the actions of EPA, DHA and their metabolites. Nevertheless, since a number of chronic diseases, including osteoporosis, involve an inflammatory component, there is biological plausibility that n-3 PUFA may modulate inflammation and thus their development and progression.

2.4.2 Flaxseed, Flaxseed Lignan, Flaxseed Oil and Bone Metabolism

There are only two in vitro studies in which effects of lignans and ALA on bone have been studied. These studies have shown that lignans and ALA have direct effects on osteoblast-like cells by increasing cell viability, DNA content, alkaline phosphatase activity, and gene expression of markers of bone formation including osteocalcin, osteonectin and type 1 collagen (Table 2-5) (Fujimori et al., 1989; Feng et al., 2008). While these in vitro studies have demonstrated the direct effects of lignan metabolites and ALA on bone cells in culture conditions, it is unknown whether these metabolites are accessible to bone tissue in vivo and thus, may modulate bone metabolism through direct local action.

2.4.2.1 Feeding Intervention Trials in Aging Animal Models

Currently, there is no research that has investigated the effects of whole FS on BMD, structure or strength in a postmenopausal osteoporotic model. However, a study by Boulbaroud et al (2008) studied the effects of 7% or 10% FS oil diet on serum markers of bone formation (alkaline phosphatase) and bone resorption (TRAP activity) in ovariectomized rats that were fed for 4 weeks (Table 2-6). The level of ALA in the FS oil of this study was 3.2% for the 7% FS oil diet
### Table 2-5: Effect of flaxseed (FS) components on bone cells in vitro

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>Dose</th>
<th>Treatment Duration</th>
<th>Results for Cell Viability, Protein mRNA or Protein activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG-63 osteosarcoma cell line</td>
<td>ED or EL</td>
<td>0.01-10 mg/mL, or 0.0 mg/mL (control)</td>
<td>2-7 days</td>
<td>• Higher cell viability, phosphatase activity, mRNA of osteocalcin, osteonectin and type I collagen at low doses</td>
<td>(Feng et al., 2008)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Lower cell viability, alkaline phosphatase activity, mRNA of osteocalcin, osteonectin and type I collagen at high doses</td>
<td></td>
</tr>
<tr>
<td>MC3T3-E1 mouse calvaria osteoblastic cell line</td>
<td>ALA</td>
<td>$10^{-6}$-$10^{-4}$ M, or 0M (control)</td>
<td>2-4 days</td>
<td>• Higher DNA content at $10^{-5}$ and $10^{-4}$ M versus control</td>
<td>(Fujimori et al., 1989)</td>
</tr>
</tbody>
</table>

ALA = α-linolenic acid, DNA = deoxyribonucleic acid, ED = enterodiol, EL = enterolactone, mRNA = messenger ribonucleic acid.
<table>
<thead>
<tr>
<th>Animal Type, (Age During Experimental Period)</th>
<th>Treatment</th>
<th>Dose</th>
<th>Control group</th>
<th>Treatment duration</th>
<th>Results for BMD, BMC, strength or biochemical markers of bone turnover</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>OVX mice (6-38 wks)</td>
<td>Ground FS</td>
<td>10% of diet</td>
<td>0% (basal diet)</td>
<td>25 weeks</td>
<td>• Higher peak load and stiffness at femur midpoint versus control group</td>
<td>(Power et al., 2007)</td>
</tr>
<tr>
<td>OVX rats (12-16 wks)</td>
<td>FS Oil</td>
<td>7% or 10% diet</td>
<td>0% (basal diet)</td>
<td>4 weeks</td>
<td>• Lower ALP and TRAP activity compared to negative control with 10% FS oil</td>
<td>(Boulbaroud et al., 2008)</td>
</tr>
<tr>
<td>OVX mice (6-35 wks)</td>
<td>ED or EL by s.c. injections</td>
<td>10 mg/kg body weight</td>
<td>Vehicle Injection</td>
<td>22 weeks</td>
<td>• No differences in BMD, BMC, or strength at femur and lumbar spine versus control</td>
<td>(Power et al., 2006)</td>
</tr>
<tr>
<td>Female rats (0-19 wks)</td>
<td>SDG</td>
<td>5% or 10% of FS diet equivalent</td>
<td>0% (basal diet)</td>
<td>PND 132</td>
<td>• No differences in BMD or femur strength</td>
<td>(Ward et al., 2001b)</td>
</tr>
</tbody>
</table>

BMC= bone mineral content, BMD= bone mineral density, ED= enterodiol, EL= enterolactone, OVX= ovariectomized, PND= postnatal day, s.c.= subcutaneous, SDG= secoisolariciresinol diglycoside, TRAP= tartrate-resistant acid phosphatase.
and 4.6% for the 10% FS oil diet, which are equivalent to approximately a 14% and 20% whole FS diet, respectively. After 4 weeks of treatment, serum alkaline phosphatase and TRAP activity in rats fed the 7% FS oil diet was similar to the ovariectomized control group (Boulbaroud et al., 2008). The greatest effects on serum levels of bone markers were observed in the rats fed the 10% FS oil diet whereby both serum alkaline phosphatase and serum TRAP activity were significantly lower than the ovariectomized control group. These results suggest that a high ALA diet may be protective against the loss of bone in ovariectomized rats and may play a role in suppressing bone loss in a postmenopausal situation. However, longer term studies are needed to determine whether a high ALA diet could also be protective to BMD, bone structure and ultimately bone strength.

Using ovariectomized athymic mice with human breast cancer, Power et al (2007) observed higher peak load and stiffness at the femur midpoint compared to control mice after a twenty-five week feeding of a 10% ground FS diet, suggesting that FS induces beneficial effects on cortical-rich bone in this mouse model of postmenopausal breast cancer (Table 2-6) (Power et al., 2007). However, using the same experimental model, Power et al (2006) did not observe similar benefits to the femur after twenty-two weeks of daily injections with EL or ED (Table 2-6) (Power et al., 2006). Thus, other components of FS (e.g. ALA and/or other lignan metabolites) may be responsible for the protective effects of FS on bone strength in ovariectomized athymic mice with human breast cancer.

### 2.4.2.2 Epidemiological Studies and Feeding Intervention Trials in Humans

To date, only one epidemiological study has examined the role of the lignan metabolite, EL, in postmenopausal osteoporosis (Table 2-7) (Kim et al., 2002). In this study, greater urinary excretion of EL was correlated with higher BMD at the vertebrae, femur neck, and Ward’s
<table>
<thead>
<tr>
<th>Study Type</th>
<th>Sample Size, Mean Age in Years (Range)</th>
<th>Treatment</th>
<th>Dose</th>
<th>Treatment Duration</th>
<th>Results for BMD, BMC, or biochemical markers of bone turnover</th>
<th>Reference</th>
</tr>
</thead>
</table>
| Cohort Study                   | • 564 postmenopausal women not on HT, 74.0  
• 326 postmenopausal women on HT, 69.4  
• (total age range, 45-90)  | Dietary LA:ALA| N/A          | N/A                | • Negatively associated with BMD at hip                                                                  | (Weiss et al., 2005)       |
| Cross-Sectional Study          | 75 postmenopausal women, 57.6 yrs (52-65)                                                             | Urinary EL    | N/A          | N/A                | • Positively associated with BMD at vertebra, femur neck, Ward's Triangle                             | (Kim et al., 2002)          |
| Cohort Study                   | 63 postmenopausal women, 63 (range not provided)                                                       | Urinary EL    | N/A          | N/A                | • Not associated with BMD at radius  
• Positively associated with rate of bone loss                                                        | (Kardinaal et al., 1998)   |
| Randomized, double-blind cross-over trial | • 20 men, 48.6 (range not provided)  
• 3 postmenopausal women, 58.3 (range not provided)                     | ALA diet      | 6.5% ALA     | 6 weeks           | • Lower NTX in ALA group versus control group  
• NTX positively associated with TNF-α                                                                 | (Griel et al., 2007)       |
### Table 2-7 (continued): Effect of flaxseed (FS) or its components on bone health in humans

<table>
<thead>
<tr>
<th>Study Type</th>
<th>Sample Size, Mean Age in Years (Range)</th>
<th>Treatment</th>
<th>Dose</th>
<th>Treatment Duration</th>
<th>Results for BMD, BMC, or biochemical markers of bone turnover</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RCT</td>
<td>179 menopausal women, 54.0 (45-64) for FS group, 55.4 (45-65) for placebo Group</td>
<td>FS muffin or wheat germ placebo muffin</td>
<td>40 g per Day</td>
<td>12 months</td>
<td>• No differences in BMD at lumbar vertebrae and femur neck</td>
<td>(Dodin et al., 2005)</td>
</tr>
<tr>
<td>RCT</td>
<td>44 postmenopausal women, 52.6 (range not provided) for FS group, 52.7 (range not provided) for placebo group</td>
<td>FS muffin or wheat flour placebo muffin</td>
<td>25 g per Day</td>
<td>16 weeks</td>
<td>• No differences in serum alkaline phosphatase or urinary deoxypyridinoline</td>
<td>(Brooks et al., 2004)</td>
</tr>
<tr>
<td>RCT</td>
<td>36 postmenopausal women, 54.0 (range not provided) for FS group, 55.0 (range not provided) for placebo group</td>
<td>Ground FS or wheat-based placebo</td>
<td>40 g per Day</td>
<td>3 months</td>
<td>• No differences in serum alkaline phosphatase, TRAP activity, urinary deoxypyridinoline or helical peptide</td>
<td>(Lucas et al., 2002)</td>
</tr>
</tbody>
</table>

ALA= α-linolenic acid, BMC= bone mineral content, BMD= bone mineral density, EL= enterolactone, HT= hormone replacement therapy, LA= linoleic acid, NTX= N-telopeptides of type I collagen, TRAP: tartrate-resistant acid phosphatase, TNF-α: tumor necrosis factor-α, N/A= not applicable.
triangle in Korean postmenopausal women with existing osteoporosis. It is important to make the distinction that these women had osteoporosis as other studies of postmenopausal women that are discussed and included in Table 2-7 include women without osteoporosis or low BMD. In healthy Dutch postmenopausal women with low (≤ 0.5%) or high (≥ 2.5%) rates of cortical bone loss at the radius (but no diagnosis of osteoporosis), greater urinary EL was correlated with greater rates of bone loss (Kardinaal et al., 1998). The reasons for discrepancies in the findings reported in these studies may involve a number of factors including variations in subject populations and methods of EL analyses. Urinary EL may also have been a marker of other unidentified dietary or lifestyle factors that are associated with BMD and the rate of cortical bone loss. Using the database from the Rancho Bernardo Study, a higher dietary linoleic acid to ALA ratio was associated with a lower hip BMD in healthy postmenopausal women not using hormone replacement therapy (~74.0 years of age), and postmenopausal women using hormone replacement therapy (~69.4 years of age) (Weiss et al., 2005). This inverse relationship was also observed at the lumbar spine in the postmenopausal women not using therapy. Thus, ALA may be useful in attenuating bone loss. However, further studies are needed to confirm whether ALA can indeed attenuate bone loss or, if its potential protective action on bone is mediated through conversion of ALA to its longer-chain products, EPA and DHA, or the result of the ratio of n-6 to n-3 PUFA.

Three randomized, double-blind, placebo-controlled trials have investigated whether FS can modulate BMD or biochemical markers of bone metabolism in healthy postmenopausal women (Table 2-7) (Lucas et al., 2002; Brooks et al., 2004; Dodin et al., 2005). The trial by Dodin et al (2005) consisted of 12 months daily treatment of 40 g FS or wheat germ (placebo) in the form of bread or ground grains. In this trial, no changes in BMD at the LV or femur neck
were observed from baseline or between the FS and wheat germ placebo groups. The two other studies consisted of 3-4 months daily treatment of 25 g or 40 g FS in the form of a muffin (Brooks et al., 2004) or ground seed (Lucas et al., 2002), respectively. In these studies by Brooks et al (2004) and Lucas et al (2002), no changes in serum markers (alkaline phosphatase, TRAP activity) or urinary markers (deoxypyridinoline) of bone metabolism were observed from baseline or between the FS and wheat flour/wheat-based placebo groups (Lucas et al., 2002; Brooks et al., 2004). Thus, these above-mentioned studies demonstrate that FS does not modulate bone metabolism or BMD in healthy postmenopausal women with normal BMD. In addition to its effects on biochemical markers of bone metabolism, Brooks et al (2004) observed a significant increase in urinary 2-hydroxyestrone to 16α-hydroxyestrone ratio with FS treatment from baseline (Table 2-7). The increase in 2-hydroxyestrone to 16α-hydroxyestrone ratio after FS treatment was also correlated with urinary lignan excretion suggesting that FS alters estrogen metabolism to favor a potentially antiestrogenic environment. However, 2-hydroxyestrone has previously been shown to exert neither estrogenic nor antiestrogenic action in hFOB/ER9 human fetal osteoblastic cells expressing high levels of the ER-α (Robinson et al., 2000). No differences in urinary estrogen metabolites (2-hydroxyestrone, 16α-hydroxyestrone) were observed between the FS and placebo groups (Brooks et al., 2004) and no differences in serum hormones (estradiol, estrone, estrone sulfate) have been observed (Lucas et al., 2002; Brooks et al., 2004) suggesting that the potential modulation of FS on estrogen metabolism may be too weak to induce changes in hormone levels and markers of bone metabolism.

Griel et al. (2007) used a combination of walnut oil and FS oil to provide ALA to 20 overweight and hypercholesterolemic men (48.6 years of age) and 3 overweight and hypercholesterolemic women (58.3 years of age) (Table 2-7). Subjects were randomized to an
Average American Diet (linoleic acid/ALA = 9.5/1), Linoleic Diet (linoleic acid/ALA = 3.5/1), or ALA Diet (linoleic acid/ALA = 1.6/1) for six weeks in a double-blind cross-over trial (Griel et al., 2007). At the end of this study, serum NTX were significantly lower following the ALA Diet compared to the Average American Diet, while no differences in bone-specific alkaline phosphatase were observed, suggesting that a diet high in ALA may support bone health by decreasing bone breakdown. In addition, the ALA Diet resulted in significantly lower serum TNF-α, a pro-inflammatory cytokine and osteoclastogenic factor, relative to the Average American Diet suggesting that a high ALA diet may attenuate osteoclastic activity and thus the resorption of bone. Since the subjects were 20 men and 3 postmenopausal women, the results from this study must be interpreted with caution. In addition, the study by Griel et al (2007) was too short in duration to measure differences in BMD. Whether a longer trial results in higher BMD, a surrogate measure of fracture risk, is unknown. It is important to note that some of these above-mentioned human trials are limited by their small sample sizes and healthy populations, thus larger trials using osteoporotic populations are warranted to determine the ability of FS to modulate bone metabolism in the postmenopausal osteoporotic situation.

2.4.3 Adverse Effects of FS

The greatest concern of FS consumption is the potential for SDG and its lignan metabolites to induce estrogenic effects on tissues such as the uterus, which may result in an increased risk for carcinogenesis. Moreover, FS accumulates cadmium (Angelova et al., 2004; Hocking & McLaughlin, 2006), a heavy metal that can also induce estrogenic effects in uterine tissue (Johnson et al., 2003). In the case of EL, it has been shown to exert estrogenic action in the uterus of transgenic estrogen-sensitive reporter mice, however, these effects were transient (Penttinen et al., 2007). However, a number of in vivo studies have reported that dietary FS and
its components do not stimulate crude markers of organ toxicity such as wet weight uterine tissue (Chen et al., 2003; Chen et al., 2006; Bergman Jungestrom et al., 2007; Chen et al., 2007; Chen et al., 2011). Since wet organ weight does not infer safety, future histological examination of tissue is needed to more fully assess the long-term safety of a food component.

2.4.4 Recommended Intakes

There are currently no dietary recommendations for the intake of FS, its lignans or its oil. However, ALA has a current adult recommendation of 1.1-1.6 g per day as set out in the Daily Reference Intakes by the Institute of Medicine (Food and Nutrition Board, 2005). A 10% FS level is used in many feeding studies because it can be attained in the human diet alone and represents an intake of approximately 25-50 g or 2½-5 tablespoons of FS per day. Thus, the ALA intake in a 10% FS diet is equivalent to 4-6 g per day which exceeds current dietary recommendations. Indeed, FS consumption is very popular in Western societies. FS oil is one of the ten most commonly used natural health products in Canada (Singh & Levine, 2006) and lignans such as SDG found in FS are ubiquitous in our food supply as well as in herbal and other nonvitamin and nonmineral natural health products (Mazur, 1998; Mazur et al., 1998; Nurmi et al., 2003; Thompson et al., 2007). Since consumption of FS and its components have been reported to be relatively safe and may prevent the development and progression of a number of chronic diseases, it is likely its use will continue to increase.

2.4.5 Summary

It is evident that FS and its components induce a wide range of effects on bone metabolism that can be in part attributed to the timing of feeding and variations in treated populations. From present literature, favorable outcomes on bone appear to be attributed to ALA rather than the lignans in FS since a greater consistency exists on the effects of high ALA diets. Nevertheless,
insufficient data on the role of FS on the aging skeleton, combined with its widespread consumption in the Western population reinforce the need for well designed trials to determine how FS may modulate bone metabolism, particularly in postmenopausal osteoporosis.

2.5 Overall Summary

HT is an effective agent for the management of bone loss during osteoporosis due to its ability to suppress bone turnover however, concerns of its long-term safety have motivated the development of new therapies that may preserve bone mass and bone strength while inducing fewer side effects. Newer formulations of HT designed with lower doses of estrogen and with a transdermal delivery system may not require the addition of progesterone in women with an intact uterus. Indeed, evidence is accumulating which demonstrates that these lower doses of estrogen monotherapy preserve bone in postmenopausal women and may be associated with fewer side effects.

In addition, combining nutritional intervention with pharmacological agents to augment health during and after menopause is increasing among women. FS is increasingly common in the Western diet, in part because of its potential therapeutic applications in the aging population. These applications include the prevention and treatment of hormone-related diseases such as osteoporosis. While many postmenopausal women consume FS, its role alone or when combined with lower doses of HT on postmenopausal bone loss has not yet been defined. The lignan metabolites produced from FS consumption contain estrogenic and antiestrogenic properties due to their structural similarity to endogenous estrogen and, the metabolites of n-3 PUFA from FS consumption have anti-inflammatory-like properties due to their ability to compete with the metabolism of inflammatory-inducing n-6 PUFA. Whether lignans and ALA
from FS consumption are accessible to skeletal tissue and thus, may exert direct action on bone turnover alone, or combined with lower doses of estrogen therapy, is unknown. In addition, it is unknown whether FS combined with lower doses of estrogen therapy may modulate the effect of estrogen therapy on uterus health. Thus, it is important to investigate the effects and mechanisms of FS, alone or combined with lower doses of estrogen therapy, on the deterioration of bone tissue and uterus health in the postmenopausal osteoporotic situation.

2.6 The Ovariectomized Rat Model of Postmenopausal Osteoporosis

The ovariectomized rat model of postmenopausal osteoporosis is an established and reproducible preclinical model for investigating the pathogenesis and progression of osteoporosis. In fact, the United States Food and Drug administration (FDA) states that the ovariectomized rat and a second, non-rodent animal model must be used for the study of agents used in the prevention or treatment of postmenopausal osteoporosis (U.S. Food and Drug Administration, 1994). Although osteoporosis occurs naturally only in humans and nonhuman primates, ovariectomy of the rat induces bone loss similar to postmenopausal women in that there is an initial rapid loss of bone, followed by a slower phase of bone loss. In addition, the rat skeleton experiences similar sequential trabecular bone remodeling in the secondary spongiosa as the human skeleton in that the remodeling cycle initiates with osteoclast activation and proceeds with resorption, reversal and then formation (Baron et al., 1984). The remodeling cycle in the rat trabecular skeleton occurs over a period of one to one and a half months (approximately 40 days) which is shorter compared to human trabeculae (100-200 days per cycle) (Baron et al., 1984; Kimmel et al., 1990; U.S. Food and Drug Administration, 1994). Thus, a three-month study of bone metabolism utilizing rat models is equivalent to approximately a one-year clinical trial.
The ovariectomized rat provides valuable information regarding the therapeutic and toxicological potential of various drugs and nutrition therapies on skeletal health because (a) it mimics postmenopausal trabecular bone loss when examined over a short period of time (12 months or less), (b) there is extensive literature on bone physiology in the ovariectomized rat and the effects of various pharmacological and dietary compounds, (c) rats have a shorter lifespan thus are good for aging studies, and (d) rats are cost-effective (Iwaniec & Turner, 2008). Rats are also able to convert FS lignans into the mammalian lignans, ED and EL (Rickard et al., 1996; Liu et al., 2006).

As with all animal models, the ovariectomized rat model has limitations to consider. One limitation is that rats lack haversian systems that are present in human cortical bone (Turner, 2001). Since haversian systems are structural units within cortical bone whereby bone remodeling takes place, the effects of various treatments on cortical bone remodeling cannot be fully elucidated in the ovariectomized rat model. Another limitation is that in some bones of female rats, growth is a lifelong process because the growth plates often do not fuse until later in life (21 months of age for lumbar vertebrae) (Jee & Yao, 2001). Growth plates at other skeletal sites such as the distal tibia and the diaphysis of long bones fuse at earlier ages (3 months and 10 months, respectively) (Jee & Yao, 2001). In addition, longitudinal bone growth continues up to 6-12 months of age and stops around 18 months of age (Kalu, 1991; Kimmel, 1991; Li et al., 1991). Thus, older rats are ideal to study bone metabolism during aging. However, due to the low accessibility to virgin aging female rats (retired breeders are often avoided because substantial bone loss occurs with multiple breeding) and high costs of aging rats in-house prior to starting a study, 3-month-old rats are often used for investigating ovariectomy-induced effects of various treatments on bone metabolism as a model of bone loss during aging. The consequence
of utilizing 3-month-old ovariectomized rats is that the effect of ovariectomy can be a suppression of bone growth rather than the stimulation of bone loss (Francisco et al., 2010), the latter which is observed in the human postmenopausal situation. Notwithstanding, it may be suitable to utilize younger rats to study the effects of estrogen-related compounds as skeletal tissue of both young (5 months old) and old (19 months old) female ovariectomized rats have been shown to respond similarly to estrogen treatment (Thompson et al., 1995).

Ovariectomy-induced weight gain that occurs in rats is believed to protect against the loss of bone strength therefore, this model is often viewed as a model for osteopenia rather than osteoporosis (Turner, 2001). However, recent investigation in our laboratory demonstrated that higher food intake, body weight and fat mass in ovariectomized rats fed ad-libitum for 14 weeks do not result in higher BMD or strength at the LV or femurs compared to their pair-fed counterparts (Jiang et al., 2008). Thus, the ovariectomized rat maintains to be a useful model of postmenopausal osteoporosis. Moreover, our work (Jiang et al., 2008) demonstrates pair-feeding of ovariectomized rats and sham controls is not essential for skeletal research in studies of three months in duration.
CHAPTER 3
Hypotheses and Objectives
3.0 Hypotheses and Objectives

3.1 Hypotheses

**Overall Hypothesis:** FS will protect against deterioration of bone tissue and will enhance the protective effect of lower doses of estrogen therapy (LD or ULD) by reducing bone resorption and bone turnover, while exerting no adverse effects on uterus health in the ovariectomized rat model of postmenopausal osteoporosis.

**Specific Hypotheses:**

Using the ovariectomized rat model of postmenopausal osteoporosis,

1. FS, alone or combined with estrogen therapy (LD or ULD), will better preserve BMD, three-dimensional bone structure and biomechanical bone strength compared to estrogen therapy alone (Chapters 4 and 5).

2. FS feeding will result in higher n-3 PUFA and lignans in the skeletal tissue (Chapters 4, 5 and 6).

3. FS, alone or combined with estrogen therapy, will preserve BMD, three-dimensional bone structure and biomechanical bone strength by reducing bone resorption and bone turnover (Chapter 7).

4. FS, alone or combined with estrogen therapy, will not adversely affect uterine morphology or uterine cell proliferation compared to estrogen therapy alone (Chapter 8).
3.2 Objectives

**Overall Objective:** To determine the effects and mechanisms of FS, alone or combined with lower doses of estrogen therapy (LD, ULD), on deterioration of bone tissue and uterus health by measuring a comprehensive set of outcomes in the ovariectomized rat model of postmenopausal osteoporosis.

**Specific Objectives:**

Using the ovariectomized rat model of postmenopausal osteoporosis,

1. determine the effect of FS, alone or combined with estrogen therapy (LD or ULD), on BMD, three-dimensional bone structure, and biomechanical bone strength (Chapters 4 and 5).

2. determine the effect of FS feeding on skeletal fatty acid and lignan compositions (Chapters 4, 5 and 6).

3. determine mechanisms whereby FS, alone or combined with estrogen therapy, may modulate bone metabolism (Chapter 7).

4. determine the effects of FS, alone or combined with estrogen therapy, on uterine morphology and uterine cell proliferation (Chapter 8).
3.3 Scientific Approach

To test the effects and mechanisms of FS, alone or combined with lower doses of estrogen therapy, on deterioration of bone tissue and uterus health in a postmenopausal osteoporotic situation, a comprehensive set of outcomes can be measured in both bone and uterine tissues (Figure 2-9).

The first study (Chapter 4) was designed to determine the effects of FS, alone and combined with LD, on bone quantity, bone quality and bone strength using DEXA, microcomputed tomography (µCT) and biomechanical strength testing, respectively. BMD was chosen to measure bone quantity because it is used in the clinical setting to diagnose osteoporosis (Papaioannou et al., 2010). Bone volume fraction, trabecular number, trabecular thickness and trabecular separation were chosen to measure bone quality because they are related to bone strength (Roux et al., 2010; Stein et al., 2010) and they provide a useful three-dimensional assessment of trabecular bone morphology which cannot be done with histomorphometry (Bouxsein et al., 2010). Peak load obtained by biomechanical strength testing was chosen as the measure for bone strength because it measures the amount of force a bone can withstand before fracture and thus is a surrogate measure of fracture risk. The second study (Chapter 5) was designed to determine whether FS antagonizes the effect of an even lower dose of estrogen (ULD) on bone quantity and bone strength using DEXA and biomechanical strength testing, respectively.

Study 1 and Study 2 were also designed to determine whether ALA can be incorporated into skeletal tissue after FS intake and was determined by gas chromatography, while Study 3 (Chapter 6) was designed to determine whether lignan can be incorporated into skeletal tissue by scintillation counting of tritium in skeletal tissue of rats gavaged tritium-labeled SDG. Gas
Figure 3-1: Scientific approach

Study 1 (Chapter 4) was designed to determine the effects of flaxseed (FS), alone and combined with low-dose estrogen therapy (LD) on bone quantity, bone quality and bone strength using dual energy x-ray absorptiometry (DEXA), microcomputed tomography (µCT) and biomechanical strength testing, respectively. Study 2 (Chapter 5) was designed to determine whether FS antagonizes the effect of an even lower dose of estrogen (ULD) on bone quantity and bone strength using DEXA and biomechanical strength testing, respectively. Study 1 and Study 2 both determined whether skeletal tissue directly incorporates ALA after FS intake using gas chromatography, while Study 3 (Chapter 6) was designed to determine whether skeletal tissue directly incorporates lignans by scintillation counting of tritium in skeletal tissue of rats gavaged tritium-labeled lignan secoisolaricresinol diglycoside (SDG). Study 4 (Chapter 7) was designed to determine how bone resorption, bone formation and overall bone turnover are modulated by FS combined with estrogen therapy using enzyme-linked immunosorbent assay (ELISA) (TRAP-5β, CTX) and Luminex xMAP technology (CTX) in serum and by using immunohistochemical analyses (TRAP, OPG, RANKL, osterix, osteocalcin) on decalcified vertebrae sections. Study 5 (Chapter 8) was designed to determine the effects of FS, alone and combined with HT, on markers of uterine health using crude weight measurements, qualitative morphologic analysis using hematoxylin and eosin staining of uterine sections, and immunohistochemical analysis of cell proliferation in the endometrial epithelium. To carry out these studies, all experiments were completed using the established ovariectomized rat model of postmenopausal osteoporosis.
Chromatography was chosen to determine fatty acid composition because it is a reliable technique for the separation of lipids in skeletal tissue (Watkins et al., 2000). Scintillation counting of tritium-labeled SDG metabolites was chosen to determine accessibility of lignans to bone since a standardized technique to extract SDG metabolites in tissue has not yet been determined. Study 4 (Chapter 7) was designed to determine how bone resorption, bone formation and bone turnover are modulated by FS combined with estrogen therapy using enzyme-linked immunosorbent assay (ELISA) (TRAP-5β, CTX) and Luminex xMAP technology (CTX) in serum and by using immunohistochemical analyses (TRAP, OPG, RANKL, osterix, osteocalcin) on decalcified vertebrae sections. TRAP and osterix were analyzed as markers of osteoclast and osteoblast numbers, respectively, while CTX and osteocalcin were analyzed as markers of osteoclast and osteoblast activities, respectively. OPG and RANKL were analyzed because they play a pivotal role on the formation, activity and survival of osteoclasts (Khosla, 2001) and, postmenopausal osteoporosis is characterized by an imbalance in bone remodeling whereby bone resorption supersedes bone formation. Both serum and bone immunohistochemical analyses were implemented in Study 4 since serum is sensitive to early changes in bone remodeling while immunohistochemical analyses on decalcified sections of bone provide site-specific information of the treatment-induced effects on bone turnover. Study 5 (Chapter 8) was designed to determine the effects of FS, alone and combined with HT, on markers of uterine health using crude weight measurements, qualitative morphologic analysis using hematoxylin and eosin staining of uterine sections, and immunohistochemical analysis of cell proliferation in the endometrial epithelium. To carry out these studies, all experiments were completed using the established ovariectomized rat model of postmenopausal osteoporosis.
CHAPTER 4
Study 1

4.0 Flaxseed combined with low-dose estrogen therapy preserves bone tissue in ovariectomized rats

4.1 Introduction

Postmenopausal osteoporosis is a skeletal disease characterized by compromised bone strength, predisposing an individual to an increased risk of fractures (NIH Consensus Development Panel on Osteoporosis Prevention Diagnosis and Therapy, 2001). As discussed in Section 2.3, estrogen therapy reduces the risk of fragility fractures however, findings from the WHI demonstrated that its use is associated with a number of adverse effects (Anderson et al., 2004).

In light of a number of limitations to the WHI trial and, considering that estrogen therapy is effective in protecting against menopausal-related conditions including bone loss, lower doses of estrogen therapy are currently being tested to determine whether they can provide the same effectiveness as standard dose estrogen therapy but with potentially fewer side effects (Richman et al., 2006).

It is also important to consider that many women are using dietary supplements for therapeutic support for menopausal symptoms and related conditions including osteoporosis (Mahady et al., 2003; Chong et al., 2007). FS is an example of a commonly consumed food that is increasingly available in the Western Diet and has the potential to modulate bone health through the action of its bioactive components, ALA and mammalian lignans (i.e. ED and EL) (reviewed in Section 2.4.2). However, a paucity of data exists on the potential effects of FS or its components on bone metabolism, particularly in the prevention or treatment of postmenopausal osteoporosis and, conflicting results on bone health have been reported (Kardinaal et al., 1998; Kim et al., 2002; Lucas et al., 2002; Brooks et al., 2004; Dodin et al., 2005; Griel et al., 2007). For example, one epidemiological study observed a positive
association between urinary EL and BMD in postmenopausal osteoporotic and osteopenic women (Kim et al., 2002) while in randomized, double-blinded, placebo-controlled trials, short-(3-4 months) and long-term (12 months) supplementation of FS did not alter biochemical markers of bone metabolism or BMD in healthy menopausal women (Lucas et al., 2002; Brooks et al., 2004; Dodin et al., 2005). It is worthy to note that epidemiological data does not infer cause and effect and that the later studies exclusively used healthy postmenopausal women in their designs. Animal studies using a number of diseased models not including osteoporosis (e.g. inflammatory bowel disease, breast cancer, polycystic kidney disease) have also reported conflicting findings whereby FS or its components have induced weak beneficial, detrimental or no effect on bone metabolism (Cohen et al., 2005; Power et al., 2006; Power et al., 2007; Weiler et al., 2007). Thus, it is of interest to investigate whether FS may modulate bone metabolism in a postmenopausal osteoporotic situation.

The intake of FS and its components is widespread in the Western population. In fact, the number of food products containing FS has tripled since 2003 (Fitzpatrick, 2007). FS oil is also one of the ten most commonly used natural health products in Canada (Singh & Levine, 2006). Lignans are derived from seeds, tea, coffee, wines, whole-grain cereals, berries, beans and other fruits and vegetables, and, they are ubiquitously present in herbal and other nonvitamin and nonmineral natural health products (Mazur, 1998; Mazur et al., 1998; Thompson et al., 2007). More importantly, Western women who take prescription or over-the-counter drugs such as HT are more likely to consume dietary supplements, many of which contain phytoestrogens (Mahady et al., 2003; Singh & Levine, 2006). Since n-3 PUFA, the mammalian lignans and estrogen therapy may modulate similar mechanisms in bone metabolism, the effects of their combination may differ than when these compounds are administered alone.
Therefore, the objectives of the study were to determine, using the well-established FDA-approved ovariectomized rat model of osteoporosis (U.S. Food and Drug Administration, 1994), the effects of FS, alone or combined with LD, on BMD, three-dimensional bone structure and biomechanical bone strength at the LV. The LV was chosen as the primary skeletal site of interest because it contains a greater proportion of metabolically-active trabecular bone than the femur and thus is often more responsive to the cessation of estrogen production and potentially to interventions. The BMD and biomechanical strength of long bones (femur and tibia) were considered as secondary outcomes. Feeding fish oil, containing long chain PUFA has been shown to result in higher levels of EPA and DHA (Watkins et al., 2005) but whether FS feeding results in greater incorporation of ALA, EPA and DHA has not been determined. Thus, another objective of the present study was to determine the effect of FS, alone or combined with LD, on skeletal (LV, tibia) fatty acid composition.

4.2 Materials and Methods

4.2.1 Flaxseed Analyses

FS (Johnson Seeds Ltd., Arborg, Manitoba, Canada) was analyzed for protein, carbohydrate, fat, and total fiber contents by Maxxam Analytics Inc. (Mississauga, Ontario, Canada) according to the standard methods of the Association of Official Analytical Chemists (AOAC, 1997). Lignans were analyzed as previously described (Thompson et al., 2006): 50 g of defatted FS was extracted twice with 70% methanol (25 mL) at 60-70°C for 2 hours while being shaken in a water bath. The sample was then evaporated to dryness in a rotary evaporator at 60°C under vacuum. 5 mL of 1 M sodium hydroxide was then added to the dry residue for 3 hours at room temperature after which it re-dissolved in 5 mL of 1 M acetic acid and passed through a C18
solid-phase extraction column (SPE column, Octadecyl C18/14%, 200 mg/3 mL; Applied Separations, Allentown, PA, USA) that was first preconditioned with 5 mL methanol/chloroform (1:1, v/v), followed by 5 mL methanol and then 5 mL distilled water. The column was then washed with 5 mL distilled water then eluted with 4 mL methanol, and evaporated using a vacuum rotary evaporator under reduced pressure at 60°C. After complete evaporation, 5 mL of 0.1 M sodium acetate buffer and 50 µL of β-glucuronidase was added to the residue and incubated overnight in a 37°C water bath. A solid-phase extraction was performed once again on the residue to remove the enzyme from the sample and once the eluent was evaporated to dryness, the sample residue was reconstituted with 1 mL methanol prior to derivatization with 300 µL of Tri-Sil Reagent (Pierce, Rockford, IL, USA). After derivatization, the sample was dissolved in 150 µL of hexane for GC-MS analysis. An Agilent 6890 series GC system (Agilent Technologies, Wilmington, DE, USA) interfaced with an Agilent 5973 network mass selective detector and with an HP-5ms (5%- phenyl) methypolysiloxane capillary column (25 m × 0.12 mmi.d. × 0.25 µm film thickness; Agilent Technologies) was used for analysis. The samples were injected in splitless mode and eluted using a temperature program set initially at 100°C and held for 1 min, then increased at 15°C/min and held at 280°C for 17 min. The carrier gas was helium at a flow rate of 1 mL/min. The total running time was 30 min. Lignans were analyzed in the scan ion monitoring mode.

To determine the fatty acid composition of the FS, FS was ground using a large capacity coffee grinder (Braun Canada Div., Mississauga, Ontario, Canada). Total lipids of the ground FS were extracted with chloroform/methanol (2:1, v/v) according to the Folch method (Folch et al., 1957). After extraction, lipids were dried under gentle nitrogen, saponified with 0.5 M of potassium hydroxide, and methylated with 14% boron trifluoride-methanol solution (Sigma-
Fatty acid methyl esters were separated using an Agilent 6890 gas chromatograph equipped with a flame ionization detector and separated on an Agilent J&W fused silica capillary column (DB-23; 30 m, 0.25 µm film thickness, 0.25 mm inner diameter; Agilent, Palo Alto, CA, USA). Samples were injected in splitless mode and fatty acid methyl esters were eluted using a temperature program set initially at 50°C and held for 2 min, increased at 20°C/min and held at 170°C for 1 min, increased at 3°C/min and held at 212°C for 10 min to complete the run. Helium was used as a carrier gas and set to a 0.7 mL/min constant flow rate. Fatty acids were identified by comparing the relative retention times with those of a known standard mixture (GLC 463; Nu-Chek-Prep, Elysian, MN, USA). The area under each peak was determined using ChemStation (Version B.01.01; Agilent).

4.2.2 Animals and Diets

Care and use of the animals were carried out in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993), and the experimental protocol was approved by the Animal Ethics Committee at the University of Toronto, Toronto, Canada. Three month-old female Sprague-Dawley rats (n=48 ovariectomized rats, mean body weight 313 ± 2 g; n=12 sham rats, mean body weight 282 ± 3 g) were purchased from Charles River Canada (St-Constant, QC, Canada), housed two per cage and maintained at 22°–24°C and 50% humidity with 12:12-hr light:dark cycle.

The basal diet (BD) was the AIN-93M (Reeves et al., 1993) formulation while the FS diet consisted of the BD supplemented with 10% freshly ground FS corrected for the contribution of FS to fat, fiber, available carbohydrates, and protein such that the diets were isocaloric. The quantity of FS used in this study is achievable in humans by consuming per day 2.5-5 tablespoons FS incorporated into 1-2 muffins or with foods such as yogurt, apple sauce and
cereals (Thompson et al., 2005; Brooks et al., 2004; Nesbitt et al., 1999). Both diets were devoid of phytoestrogens and prepared by Dyets Inc (Bethlehem, PA, USA). Fresh diet was provided every day and rats received free access to water. Food intake was measured daily and body weights were measured weekly.

### 4.2.3 Experimental Design

After 1 week acclimatization on the BD, rats were randomized into the following groups (n=12/group): (1) negative (NEG) control fed the BD; (2) FS, fed BD supplemented with 10% ground FS; (3) LD, fed BD and subcutaneously implanted with a 17β-estradiol pellet (13 µg, 90 day release; Innovative Research of America, Sarasota, FL, USA); and (4) FS+LD, fed the FS diet and implanted with a 17β-estradiol pellet. These pellets have been shown to effectively deliver estrogen in bone metabolism studies (Vanin et al., 1995; Lee et al., 2002b; McMillan et al., 2007). A sham-operated group fed the BD was included as a positive control (POS). The pellets were placed subcutaneously in the interscapular region while rats were anaesthetized with isoflurane (3%) dissolved in oxygen and analgesic buprenorphine (0.05 mg/kg body weight) was administered subcutaneously following surgery. The estrogen pellet was titrated to mimic LD monotherapy (25 µg/day transdermal estrogen monotherapy) currently prescribed to postmenopausal women (North American Menopause Society, 2010b).

At the end of the study (twelve weeks duration), rats were sacrificed by CO2 asphyxiation followed by cervical dislocation. A twelve-week study duration was chosen for the present research because in rats, it approximates a one-year clinical trial in postmenopausal women (Baron et al., 1984; Kimmel et al., 1990; U.S. Food and Drug Administration, 1994). Total food intake and final body weight determined. Femurs, tibias and LV1-3 were removed, cleaned of soft tissue, and stored at –80°C until further analyses.
4.2.4 Bone Mineral Density of Lumbar Vertebrae, Whole and Proximal Femur, and Whole and Proximal Tibia

Intact vertebrae (LV1–LV3), left femur and left tibia from each rat were placed on a plastic tray and scanned in air at room temperature using pDEXA® SABRE™ dual-energy x-ray bone densitometry (Orthometrix, White Plains, NY, USA) and a specialized software program (Lunar software version 1.46). Bones were scanned at a speed of 10 mm/s and at a resolution of 0.2 x 0.2 mm. The BMD of the femoral and tibial regions rich in trabecular bone was also determined by selecting the proximal 1/3 of the femur and tibia. The coefficient of variation for 5 scans of intact LV1-3, femur and tibia BMD were 0.77%, 0.20% and 1.01%, respectively.

4.2.5 Three-Dimensional Structure of Lumbar Vertebrae

Intact vertebrae (LV5) from each rat were placed in water and scanned in air at room temperature using microcomputed tomographical measurement (Scanco Medical, Basserdorf, Zurich, Switzerland). The x-ray source was set at a tube voltage of 70 kVp and at a tube current of 114 µA. A high resolution mode with an X, Y and Z resolution of 6 µm was used to scan the LV samples. Acquisition files were obtained at 1000 projections with 2048 samples each (per 180° rotation), 0° angle increment, 300 ms integration time, and 1 frame averaging.

800-1300 axial-cut slices that were 6µm thick created the scanned image. To evaluate the trabecular properties of the LV, a region of interest was chosen to represent 400 transverse CT slices at the midpoint of the bone after the total height of the LV between the cranial and caudal plates of the bone were measured. Trabecular properties evaluated included bone volume fraction (BV/TV, %), trabecular thickness (Tb.Th, mm), trabecular number (Tb.N, mm⁻¹), and trabecular separation (Tb.Sp, mm).
4.2.6 Biomechanical Strength Testing of Lumbar Vertebrae, Femur and Tibia

Biomechanical strength properties were determined using a materials testing system (Model 4442 Universal Testing System, Instron Corp., Canton, MA, USA) and a specialized software program (Instron Series IX Automated Materials Tester-Version 8.15.00; Instron Corp.).

Immediately before biomechanical testing, LV2, right femur and left tibia from each rat were rehydrated in physiological saline for 3 hrs at room temperature. Femur and tibia lengths were measured using an electronic precision caliper (Cederlane Laboratories Ltd., Hornby, ON, Canada) to determine their midpoint.

To determine strength properties a skeletal site rich in trabecular bone, individual LV2 were placed in the center of a stainless steel plate and a compression force to the vertebra was applied by lowering a second suspended stainless steel plate at a constant rate of 2 mm/min until compression of LV2 was achieved (Fonseca & Ward, 2004). Peak load (i.e. the maximum force a bone can withstand before fracture occurs) was identified as the first peak of the load-displacement curve.

To determine the strength properties of a skeletal site rich in cortical bone, three-point bending was performed at the femur and tibia midpoints as previously described (Fonseca & Ward, 2004; Reinwald et al., 2004). The posterior surfaces of the right femur and the lateral surfaces of the left tibia were placed on two 1-mm wide base supports with a jig span width of 15 mm so that the midpoint was positioned directly under the crosshead. The crosshead was then lowered at a speed of 2 mm/min applying force to the femur and tibia midpoint until fracture occurred to determine the peak load, the maximum force a bone can withstand until fracture.
4.2.7 Fatty Acid Composition of Lumbar Vertebrae and Tibia

LV3 and tibia were wrapped in tinfoil, snap frozen in liquid nitrogen and crushed with a hammer. Large bone masses were then pulverized by mortar and pestle and the powder was transferred to 50 mL glass screw cap tubes. Total lipids of the ground FS and bones were extracted and fatty acid compositional analysis was carried out as described in Section 4.2.1.

4.2.8 Statistical Analysis

One-way ANOVA was performed for each outcome, and the Student-Newman Keul’s post-hoc test was used to determine differences among groups. To determine whether different long bones respond similarly to FS and LD interventions, ovariectomized groups were combined and Pearson correlations were performed to determine the relationship between tibia BMC and femur BMC; and tibia midpoint peak load and femur midpoint peak load. In addition, linear regression analyses were performed on combined ovariectomized groups to determine the relationships between BMC and midpoint peak load in both the femurs and tibias. To determine whether differences in fatty acid composition are related to the observed differences in bone mineral and bone strength, linear regressions were conducted between BMD, BMC, peak load and fatty acid composition in both the LV and tibia. All statistical analyses were performed using SigmaStat (Systat Software Inc, Version 3.5, Chicago, IL, USA). Results are expressed as mean ± SEM. The significance level was set at $p < 0.05$.

4.3 Results

4.3.1 Flaxseed Lignan and Fatty Acid Composition

The proximate lignan and fatty acid composition are shown in Table 4-1. Lignan composition is expressed as mg/100g FS. The total lignan concentration in the FS was 191.29 mg per 100 g of
FS. Secoisolariciresinol was the primary lignan followed by lariciresinol, pinoresinol, and matairesinol. The equivalent SDG concentration in the FS was 339 mg/100g FS.

Fatty acid composition is reported as a percentage of total fatty acids. ALA was the primary fatty acid detected in the FS sample (63.65% of total fatty acids), followed by linoleic, palmitic, stearic, and smaller amounts of oleic and cis-vaccenic acids (Table 4-1). No detectable levels of EPA or DHA were found in the FS sample.

4.3.2 Total Food Intake and Body Weight

There were no significant differences in total food intake or final body weight among ovariectomized groups, however, POS had a significantly lower total food intake (1446 ± 30 g vs. 1566 ± 37 g) (p<0.05) and final body weight (377 ± 13 g vs. 443 ± 12 g) (p<0.001) compared to the NEG group.

4.3.3 Bone Mineral Density, Bone Mineral Content and Peak Load of Lumbar Vertebrae

At the end of the 12-week feeding trial, BMD (Figure 4-1A) and BMC (Figure 4-1B) of LV1-3 were significantly lower in the NEG group compared to POS (p<0.05). BMD was lowest in the FS and NEG groups and did not differ from each other. FS+LD resulted in a significantly higher BMD compared to both FS and NEG groups (p<0.05) while LD resulted in a BMD that was similar to the NEG, FS and FS+LD groups (Figure 4-1A). FS+LD resulted in BMC that was significantly higher than the FS group (p<0.05), and no other differences in BMC were observed (Figure 4-1B).

As expected, POS resulted in a significantly higher peak load at LV2 compared to the NEG group (Figure 4-1C). Peak load of LV2 was lowest in the FS and NEG groups and did not differ from each other, while FS+LD in a significantly higher peak load compared to NEG (p<0.01) and FS (p<0.01) groups and did not differ from the POS group. LD resulted in
Table 4-1: Flaxseed (FS) nutrient, fatty acid, and lignan composition

<table>
<thead>
<tr>
<th>Nutrient Composition (g/100g FS)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories</td>
<td>550</td>
</tr>
<tr>
<td>Fat</td>
<td>37</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0</td>
</tr>
<tr>
<td>Sodium</td>
<td>0.03</td>
</tr>
<tr>
<td>Potassium</td>
<td>0.66</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>32</td>
</tr>
<tr>
<td>Fiber</td>
<td>21</td>
</tr>
<tr>
<td>Sugars</td>
<td>1</td>
</tr>
<tr>
<td>Protein</td>
<td>22</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>&lt;5 ug</td>
</tr>
</tbody>
</table>

Fatty Acid Composition (% Total)

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Percentage ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td>C16:0</td>
<td>7.11 ± 0.03</td>
</tr>
<tr>
<td>C18:0</td>
<td>6.52 ± 0.02</td>
</tr>
<tr>
<td>C18:1 c9</td>
<td>0.46 ± 0.46</td>
</tr>
<tr>
<td>C18:1 c11</td>
<td>0.46 ± 0.46</td>
</tr>
<tr>
<td>C18:2 n6</td>
<td>21.19 ± 0.00</td>
</tr>
<tr>
<td>C18:3 n3</td>
<td>63.65 ± 0.02</td>
</tr>
<tr>
<td>C20:0</td>
<td>0.24 ± 0.00</td>
</tr>
<tr>
<td>C22:0</td>
<td>0.18 ± 0.00</td>
</tr>
</tbody>
</table>

Total Saturates 14.25 ± 0.01
Total Monounsaturates 0.92 ± 0.00
Total PUFA 84.83 ± 0.02
Total n-6 PUFA 21.19 ± 0.00
Total n-3 PUFA 63.65 ± 0.02
n-6/n-3 0.33 ± 0.00

Lignan Composition (mg/100g FS)

<table>
<thead>
<tr>
<th>Lignan</th>
<th>Amount ± SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secoisolariciresinol</td>
<td>178.73 ± 62.06</td>
</tr>
<tr>
<td>Matairesinol</td>
<td>4.16 ± 0.05</td>
</tr>
<tr>
<td>Lariciresinol</td>
<td>4.23 ± 0.84</td>
</tr>
<tr>
<td>Pinoresinol</td>
<td>4.17 ± 0.76</td>
</tr>
</tbody>
</table>

Total 191.29 ± 60.51

PUFA=polyunsaturated fatty acids. All data that are expressed as mean ± SEM are based on duplicate analysis. Single analysis of nutrient composition performed by Maxxam Analytics. The following fatty acids were not detected: C14:1 c9, C16:1 c9, C18:3 n6, C20:2 n6, C20:3 n6, C20:4 n6, C20:3 n3, C20:5 n3, C22:1 n9, C22:2 n6, C22:4 n6, C22:5 n6, C22:5 n3, C22:6 n3.
Figure 4-1: Effect of 10% dietary flaxseed (FS), low-dose estrogen therapy (LD) and their combination (FS+LD) on bone mineral density (BMD) (A), bone mineral content (BMC) (B), and peak load (C) at the lumbar vertebrae.

POS= positive control, NEG= negative control. LD= 13 µg, 90-day release. Bars with different letters indicate a significant difference among groups at p<0.05 by one-way ANOVA followed by Student-Newman Keul’s post-hoc test. Data are mean ± SEM. n= 11-12 rats/group.
LV2 peak load that did not differ significantly compared to both the POS and NEG groups (Figure 4-1C).

4.3.4 Three-Dimensional Structure of Lumbar Vertebrae

As expected, NEG resulted in BV/TV and Tb.N that were significantly lower than POS (p<0.001) and resulted in Tb.Sp that was significantly greater than POS (p<0.001) (Table 4-2). FS+LD resulted in the greatest Tb.N compared to all ovariectomized groups (p<0.01) and did not differ from the POS group. Likewise, FS+LD resulted in the lowest Tb.Sp compared to all other ovariectomized groups (p<0.05) and did not differ from the POS group. BV/TV was significantly higher in the FS+LD group compared to both the NEG (p<0.05) and FS (p<0.01) groups and tended to be higher than LD (p=0.06) (Table 4-2). No other differences were observed with BV/TV, Tb.N and Tb.Sp. There were no differences in Tb.Th among groups.

4.3.5 Bone Mineral Density, Bone Mineral Content of Whole and Proximal Region of Femur and Peak Load at the Femur Midpoint

BMD at the whole and proximal region of the femur was significantly lower in the NEG group compared to the POS group (p<0.05). Whole femur BMD did not differ among the NEG, FS, LD or FS+LD groups (Table 4-3). A similar pattern was observed in the proximal region of the femur whereby no differences in BMD occurred among ovariectomized groups in this region. There were no significant differences in BMC at the whole or proximal region of the femur and, no differences in peak load at the femur midpoint were observed among groups (Table 4-3).

4.3.6 Bone Mineral Density, Bone Mineral Content of Whole Tibia and Proximal Region of Tibia and Peak Load at the Tibia Midpoint

No significant differences in whole tibia BMD, BMC or midpoint peak load (Table 4-3) were observed among ovariectomized groups. A similar pattern was observed in proximal region of
<table>
<thead>
<tr>
<th></th>
<th>POS</th>
<th>NEG</th>
<th>FS</th>
<th>LD</th>
<th>FS + LD</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV/TV (%)</td>
<td>0.297 ± 0.015&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.202 ± 0.016&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.194 ± 0.010&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.221 ± 0.011&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.255 ± 0.012&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tb.N (mm&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>3.706 ± 0.095&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.757 ± 0.103&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.806 ± 0.088&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.075 ± 0.088&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.465 ± 0.103&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tb.Th (mm)</td>
<td>0.076 ± 0.002</td>
<td>0.074 ± 0.002</td>
<td>0.074 ± 0.001</td>
<td>0.075 ± 0.002</td>
<td>0.075 ± 0.001</td>
</tr>
<tr>
<td>Tb.Sp (mm)</td>
<td>0.254 ± 0.007&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.346 ± 0.016&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.348 ± 0.012&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.311 ± 0.010&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.276 ± 0.010&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

BV/TV= bone volume fraction, Tb.N= trabecular number, Tb.Th= trabecular thickness, Tb.Sp= trabecular separation, POS= positive control, NEG= negative control, LD= low-dose estrogen therapy. LD= 13 µg, 90-day release. Different letters within a row denote significant difference among groups at p<0.05 by one-way ANOVA, post-hoc Student Newman-Keul's post-hoc test. Data are mean ± SEM. n=12/group.
Table 4-3: Bone mineral density (BMD), bone mineral content (BMC), and peak load in whole and regional femurs and tibia

<table>
<thead>
<tr>
<th></th>
<th>POS</th>
<th>NEG</th>
<th>FS</th>
<th>LD</th>
<th>FS+LD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole Femur</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD (mg/cm²)</td>
<td>237.94 ± 6.43ᵃ</td>
<td>212.87 ± 2.84ᵇ</td>
<td>212.60 ± 2.37ᵇ</td>
<td>212.04 ± 3.31ᵇ</td>
<td>221.61 ± 2.43ᵇ</td>
</tr>
<tr>
<td>BMC (mg)</td>
<td>478.47 ± 19.31</td>
<td>455.71 ± 10.67</td>
<td>438.95 ± 10.70</td>
<td>439.54 ± 10.76</td>
<td>466.51 ± 10.09</td>
</tr>
<tr>
<td>Midpoint Peak Load (N)</td>
<td>161.46 ± 4.28</td>
<td>169.87 ± 5.84</td>
<td>169.84 ± 3.05</td>
<td>175.87 ± 4.61</td>
<td>177.73 ± 4.62</td>
</tr>
<tr>
<td><strong>Upper 1/3 Femur</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD (mg/cm²)</td>
<td>240.26 ± 7.18ᵃ</td>
<td>211.08 ± 3.90ᵇ</td>
<td>212.09 ± 3.57ᵇ</td>
<td>216.06 ± 4.44ᵇ</td>
<td>223.20 ± 2.79ᵇ</td>
</tr>
<tr>
<td>BMC (mg)</td>
<td>170.76 ± 7.33</td>
<td>159.04 ± 4.03</td>
<td>154.57 ± 3.92</td>
<td>155.04 ± 4.05</td>
<td>164.46 ± 3.39</td>
</tr>
<tr>
<td><strong>Whole Tibia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD (mg/cm²)</td>
<td>211.55 ± 3.73</td>
<td>199.59 ± 2.40</td>
<td>199.42 ± 3.99</td>
<td>199.70 ± 2.89</td>
<td>207.54 ± 2.11</td>
</tr>
<tr>
<td>BMC (mg)</td>
<td>362.98 ± 11.47</td>
<td>359.44 ± 8.61</td>
<td>352.15 ± 7.85</td>
<td>347.00 ± 8.80</td>
<td>371.76 ± 8.10</td>
</tr>
<tr>
<td>Midpoint Peak Load (N)</td>
<td>112.91 ± 6.34</td>
<td>120.57 ± 6.66</td>
<td>114.46 ± 5.20</td>
<td>115.34 ± 5.12</td>
<td>126.42 ± 5.42</td>
</tr>
<tr>
<td><strong>Upper 1/3 Tibia</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD (mg/cm²)</td>
<td>241.76 ± 5.63ᵃ</td>
<td>206.86 ± 1.70ᵇ</td>
<td>211.43 ± 3.48ᵇ</td>
<td>209.57 ± 3.04ᵇ</td>
<td>219.58 ± 1.82ᵇ</td>
</tr>
<tr>
<td>BMC (mg)</td>
<td>169.75 ± 6.36ᵃ</td>
<td>153.20 ± 3.26ᵇ</td>
<td>149.74 ± 3.60ᵇ</td>
<td>146.29 ± 2.95ᵇ</td>
<td>160.97 ± 3.10ᵇ</td>
</tr>
</tbody>
</table>

POS= positive control, NEG= negative control, FS= flaxseed, LD= low-dose estrogen therapy. LD= 13 µg, 90-day release. Different letters within a row denote statistical significance among groups at p<0.05 by one-way ANOVA followed by Student Newman Keul's post-hoc test. Data are expressed as mean ± SEM. n=9-12/group.
the tibia whereby the combination of FS+LD resulted in BMC that was intermediate to all groups (Table 4-3).

4.3.7 Correlation and Linear Regression Analyses: Bone Mineral Content and Peak Load

There was a significant and positive correlation between tibia and femur BMC ($r=0.915$, $p<0.001$) (Figure 4-2A), however no significant correlation between tibia and femur midpoint peak load was observed ($r=0.287$, $p=0.076$) (Figure 4-2B). Linear regression analyses showed a significant and positive relationship between BMC and midpoint peak load at both the femur ($r=0.634$, $p<0.001$, $35.805 + 0.305 \times \text{BMC}$) and tibia ($r=0.580$, $p<0.001$, $7.707 + 0.358 \times \text{BMC}$).

4.3.8 Fatty Acid Composition of Lumbar Vertebrae and Whole Tibia:

LV3 (Table 4-4) and whole tibia fatty acid composition (Table 4-5) are reported as percentage of total fatty acids. In LV3, relative levels of ALA and its long chain derivative, EPA were significantly higher in the FS and FS+LD groups compared to all other groups ($p<0.001$). Moreover, FS+LD resulted in a significantly higher EPA compared to FS ($p<0.05$) (Table 4-4). DHA was highest in the POS and FS+LD groups compared to the NEG and FS groups ($p<0.05$), while FS resulted in DHA levels similar to all groups. FS and FS+LD resulted in significantly lower levels of linoleic acid compared to all other groups ($p<0.001$), and did not differ from each other. Arachidonic acid was lowest in the FS group compared to all other groups ($p<0.05$) but did not differ from FS+LD. Total relative levels of n-3 and n-6 PUFA were higher and lower, respectively, in the FS and FS+LD groups compared to the other treatments ($p<0.001$). n-6/n-3 ratio was significantly lower in the FS and FS+LD groups compared to all other groups ($p<0.001$). While no differences in total saturates, or monounsaturates were observed, FS+LD had significantly higher total PUFAs compared to the LD group ($p<0.05$). No other differences in total PUFAs were observed among groups (Table 4-4).
Figure 4-2: Relationship between tibia and femur bone mineral content (BMC) (A) and tibia and femur midpoint peak load (B).
<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>POS</th>
<th>NEG</th>
<th>FS</th>
<th>LD</th>
<th>FS+LD</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0 (myristic)</td>
<td>1.62 ± 0.08^b</td>
<td>2.05 ± 0.08^ab</td>
<td>2.14 ± 0.07^a</td>
<td>2.05 ± 0.19^ab</td>
<td>1.84 ± 0.12^ab</td>
</tr>
<tr>
<td>C14:1 n5 (myristoleic)</td>
<td>0.33 ± 0.08</td>
<td>0.33 ± 0.04</td>
<td>0.34 ± 0.02</td>
<td>0.33 ± 0.05</td>
<td>0.34 ± 0.05</td>
</tr>
<tr>
<td>C16:0 (palmitic)</td>
<td>25.64 ± 0.25</td>
<td>26.63 ± 0.44</td>
<td>25.96 ± 0.48</td>
<td>25.99 ± 0.36</td>
<td>24.97 ± 0.31</td>
</tr>
<tr>
<td>C16:1 n7 (palmitoleic)</td>
<td>5.90 ± 0.58^b</td>
<td>8.63 ± 0.53^a</td>
<td>8.93 ± 0.40^a</td>
<td>8.93 ± 0.32^a</td>
<td>8.54 ± 0.54^a</td>
</tr>
<tr>
<td>C18:0 (stearic)</td>
<td>9.94 ± 1.50^a</td>
<td>6.18 ± 0.25^b</td>
<td>6.18 ± 0.28^b</td>
<td>6.26 ± 0.69^b</td>
<td>6.97 ± 0.46^b</td>
</tr>
<tr>
<td>C18:1 n9 (oleic)</td>
<td>27.78 ± 1.50</td>
<td>28.34 ± 0.41</td>
<td>28.22 ± 0.51</td>
<td>28.86 ± 0.56</td>
<td>27.32 ± 0.45</td>
</tr>
<tr>
<td>C18:1 n7 (cis-vaccenic)</td>
<td>3.48 ± 0.16^ab</td>
<td>3.79 ± 0.07^a</td>
<td>3.17 ± 0.07^b</td>
<td>3.74 ± 0.09^a</td>
<td>3.23 ± 0.08^b</td>
</tr>
<tr>
<td>C18:2 n6 (linoleic)</td>
<td>15.12 ± 0.61^a</td>
<td>15.92 ± 0.70^a</td>
<td>9.50 ± 0.41^b</td>
<td>15.82 ± 0.76^a</td>
<td>10.38 ± 0.36^b</td>
</tr>
<tr>
<td>C18:3 n6 (\gamma-linolenic)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C18:3 n3 (\alpha-linolenic)</td>
<td>0.95 ± 0.03^b</td>
<td>1.23 ± 0.04^b</td>
<td>8.44 ± 0.35^a</td>
<td>1.23 ± 0.07^b</td>
<td>8.30 ± 0.16^a</td>
</tr>
<tr>
<td>C20:0 (arachidic)</td>
<td>0.30 ± 0.11</td>
<td>0.12 ± 0.01</td>
<td>0.10 ± 0.02</td>
<td>0.49 ± 0.37</td>
<td>0.19 ± 0.07</td>
</tr>
<tr>
<td>C20:2 n6 (eicosadienoic)</td>
<td>0.37 ± 0.04</td>
<td>0.41 ± 0.01</td>
<td>0.29 ± 0.01</td>
<td>0.39 ± 0.02</td>
<td>0.27 ± 0.01</td>
</tr>
<tr>
<td>C20:3 n6 (eicosatrienoic)</td>
<td>0.44 ± 0.10</td>
<td>0.39 ± 0.02</td>
<td>0.30 ± 0.02</td>
<td>0.34 ± 0.03</td>
<td>0.39 ± 0.05</td>
</tr>
<tr>
<td>C20:4 n6 (arachidonlic)</td>
<td>5.21 ± 0.48^a</td>
<td>3.90 ± 0.35^b</td>
<td>2.28 ± 0.30^c</td>
<td>3.72 ± 0.36^b</td>
<td>3.00 ± 0.33^bc</td>
</tr>
<tr>
<td>C20:3 n3 (eicosatrienoic)</td>
<td>0.14 ± 0.07^b</td>
<td>0.08 ± 0.03^b</td>
<td>0.37 ± 0.02^a</td>
<td>0.12 ± 0.04^b</td>
<td>0.38 ± 0.03^a</td>
</tr>
<tr>
<td>C22:0 (behenic)</td>
<td>0.25 ± 0.11</td>
<td>0.04 ± 0.03</td>
<td>0.05 ± 0.03</td>
<td>0.04 ± 0.03</td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td>C22:5 n3 (eicosapentaenoic)</td>
<td>0.22 ± 0.08^c</td>
<td>0.44 ± 0.10^c</td>
<td>1.19 ± 0.08^b</td>
<td>0.24 ± 0.06^c</td>
<td>1.44 ± 0.08^a</td>
</tr>
<tr>
<td>C22:6 n3 (docosahexaenoic)</td>
<td>0.05 ± 0.03</td>
<td>nd</td>
<td>0.03 ± 0.03</td>
<td>0.01 ± 0.01</td>
<td>nd</td>
</tr>
<tr>
<td>C22:2 n6 (docosadienoic)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C22:4 n6 (adrenic)</td>
<td>0.93 ± 0.19</td>
<td>0.53 ± 0.06</td>
<td>0.59 ± 0.44</td>
<td>0.50 ± 0.06</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td>C22:5 n6 (docosapentaenoic)</td>
<td>0.12 ± 0.05</td>
<td>0.08 ± 0.03</td>
<td>nd</td>
<td>0.09 ± 0.03</td>
<td>nd</td>
</tr>
<tr>
<td>C22:5 n3 (docosapentaenoic)</td>
<td>0.36 ± 0.02</td>
<td>0.41 ± 0.04</td>
<td>1.22 ± 0.07</td>
<td>0.35 ± 0.03</td>
<td>1.40 ± 0.09</td>
</tr>
<tr>
<td>C22:6 n3 (docosahexaenoic)</td>
<td>0.86 ± 0.09^a</td>
<td>0.51 ± 0.05^b</td>
<td>0.70 ± 0.11^ab</td>
<td>0.48 ± 0.04^b</td>
<td>0.81 ± 0.09^a</td>
</tr>
</tbody>
</table>
Table 4-4 (continued): Fatty acid composition of LV3 expressed as percentage of total fatty acids

<table>
<thead>
<tr>
<th></th>
<th>POS</th>
<th>NEG</th>
<th>FS</th>
<th>LD</th>
<th>FS+LD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Saturates</td>
<td>37.74 ± 1.78</td>
<td>35.02 ± 0.61</td>
<td>34.43 ± 0.54</td>
<td>34.83 ± 0.93</td>
<td>33.99 ± 0.50</td>
</tr>
<tr>
<td>Total Monounsaturates</td>
<td>37.54 ± 1.96</td>
<td>41.08 ± 0.77</td>
<td>40.70 ± 0.76</td>
<td>41.87 ± 0.88</td>
<td>39.43 ± 0.90</td>
</tr>
<tr>
<td>Total PUFA</td>
<td>24.72 ± 0.47^ab</td>
<td>23.89 ± 0.75^ab</td>
<td>24.88 ± 0.93^ab</td>
<td>23.30 ± 0.71^b</td>
<td>26.58 ± 0.71^a</td>
</tr>
<tr>
<td>Total n-6 PUFA</td>
<td>22.19 ± 0.34^a</td>
<td>21.23 ± 0.77^a</td>
<td>12.96 ± 0.67^b</td>
<td>20.88 ± 0.60^a</td>
<td>14.25 ± 0.68^b</td>
</tr>
<tr>
<td>Total n-3 PUFA</td>
<td>2.52 ± 1.18^b</td>
<td>2.66 ± 0.09^b</td>
<td>11.91 ± 0.52^a</td>
<td>2.43 ± 0.14^b</td>
<td>12.32 ± 0.23^a</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>8.97 ± 0.62^a</td>
<td>8.04 ± 0.45^a</td>
<td>1.10 ± 0.07^b</td>
<td>8.72 ± 0.42^a</td>
<td>1.16 ± 0.06^b</td>
</tr>
</tbody>
</table>

PUFA = polyunsaturated fatty acid. POS = positive control, NEG = negative control, FS = flaxseed, LD = low-dose estrogen therapy. LD = 13 µg, 90-day release. Different letters within a row denote statistical significance among groups at p<0.05 by one-way ANOVA followed by Student-Newman Keul's post-hoc test. nd = not detected. Data are expressed as mean ± SEM. n=5-6/group.
In the tibia, relative levels of ALA and EPA were significantly higher in the FS and FS+LD groups compared to all other groups (p<0.001). In addition, FS+LD resulted in significantly higher ALA compared to FS (p<0.05) (Table 4-5). No differences in DHA were observed among groups. Both FS and FS+LD resulted in the lowest relative levels of linoleic acid (p<0.001) and its longer chain derivative, arachidonic acid (p<0.01), compared to the other groups. Total relative levels of n-3 and n-6 PUFA were higher and lower, respectively, in the FS and FS+LD groups compared to the other treatments (p<0.001). FS and FS+LD resulted in a lower n-6/n-3 ratio compared to all groups (p<0.001), while LD was intermediate to both the POS and NEG groups. No differences in total saturates, total monounsaturates or total polyunsaturates were observed among groups (Table 4-5).

4.3.9 Relationship Between Bone Mineral Density, Bone Mineral Content, Midpoint Peak Load and Fatty Acid Composition in Lumbar Vertebrae and Tibia

There were no significant relationships between BMD, BMC, midpoint peak load and ALA, EPA, DHA, linoleic acid, arachidonic acid, total n-3 PUFA, total n-6 PUFA or n-6/n3 ratio in both the LV-3 and tibia (data not shown).

4.4 Discussion

This study is the first to demonstrate that the combination of FS and LD results in greater protection to BMD, trabecular structure and strength at the lumbar spine compared to FS alone in this ovariectomized rat model of postmenopausal osteoporosis. Moreover, the observation that FS+LD preserved trabecular number and trabecular separation at the lumbar spine to a greater extent than LD alone indicates that FS enhances the protective effect of LD on trabecular
### Table 4-5: Fatty acid composition of tibia expressed as percentage of total fatty acids

<table>
<thead>
<tr>
<th>POS</th>
<th>NEG</th>
<th>FS</th>
<th>LD</th>
<th>FS+LD</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0 (myristic)</td>
<td>1.46 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.94 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.14 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.84 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C14:1 n5 (myristoleic)</td>
<td>0.23 ± 0.03</td>
<td>0.38 ± 0.02</td>
<td>0.37 ± 0.05</td>
<td>0.32 ± 0.03</td>
</tr>
<tr>
<td>C16:0 (palmitic)</td>
<td>21.45 ± 0.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.01 ± 0.25&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>23.54 ± 0.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.23 ± 0.44&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>C16:1 n7 (palmitoleic)</td>
<td>9.34 ± 0.79</td>
<td>12.01 ± 0.53</td>
<td>11.43 ± 0.94</td>
<td>11.02 ± 0.37</td>
</tr>
<tr>
<td>C18:0 (stearic)</td>
<td>4.62 ± 0.27</td>
<td>3.65 ± 0.10</td>
<td>3.93 ± 0.25</td>
<td>3.95 ± 0.22</td>
</tr>
<tr>
<td>C18:1 n7 (cis-vaccenic)</td>
<td>4.09 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.09 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.55 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.32 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:2 n6 (linoleic)</td>
<td>19.46 ± 1.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.81 ± 0.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.70 ± 0.53&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17.40 ± 0.88&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:3 n6 (γ-linolenic)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C18:3 n3(α-linolenic)</td>
<td>1.06 ± 0.09&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.30 ± 0.04&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.33 ± 0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.20 ± 0.07&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:4 n3 (stearidonic)</td>
<td>nd&lt;sup&gt;c&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.13 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:0 (arachidic)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C20:2 n6 (eicosadienoic)</td>
<td>0.29 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.33 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.21 ± 0.004&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.33 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:3 n6 (eicosatrienoic)</td>
<td>0.09 ± 0.05</td>
<td>0.21 ± 0.02</td>
<td>0.14 ± 0.006</td>
<td>0.18 ± 0.05</td>
</tr>
<tr>
<td>C20:4 n6 (arachidonic)</td>
<td>2.92 ± 0.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.55 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.46 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.58 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:3 n3 (eicosatrienoic)</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.20 ± 0.006&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C22:0 (behenic)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C20:5 n3 (eicosapentaenoic)</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.06 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.65 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.02 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C22:1 n9 (erucic)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C22:2 n6 (docosadienoic)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C22:4 n6 (adrenic)</td>
<td>0.47 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.39 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.12 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.42 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C22:5 n6 (docosapentaenoic)</td>
<td>0.04 ± 0.04</td>
<td>0.03 ± 0.02</td>
<td>nd</td>
<td>0.05 ± 0.03</td>
</tr>
<tr>
<td>C22:5 n3 (docosapentaenoic)</td>
<td>0.15 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.27 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.87 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.25 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C22:6 n3(docosahexaenoic)</td>
<td>0.31 ± 0.11</td>
<td>0.12 ± 0.08</td>
<td>0.40 ± 0.12</td>
<td>0.16 ± 0.10</td>
</tr>
</tbody>
</table>
Table 4-5 (continued): Fatty acid composition of tibia expressed as percentage of total fatty acids

<table>
<thead>
<tr>
<th></th>
<th>POS</th>
<th>NEG</th>
<th>FS</th>
<th>LD</th>
<th>FS+LD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Saturates</td>
<td>27.54 ± 0.70</td>
<td>28.59 ± 0.24</td>
<td>29.62 ± 0.83</td>
<td>29.02 ± 0.60</td>
<td>28.13 ± 0.35</td>
</tr>
<tr>
<td>Total Monounsaturates</td>
<td>47.68 ± 1.51</td>
<td>48.34 ± 0.62</td>
<td>47.18 ± 0.89</td>
<td>48.40 ± 0.54</td>
<td>47.15 ± 1.31</td>
</tr>
<tr>
<td>Total Polyunsaturates</td>
<td>24.79 ± 1.32</td>
<td>23.06 ± 0.59</td>
<td>23.20 ± 0.98</td>
<td>22.57 ± 0.71</td>
<td>24.72 ± 1.02</td>
</tr>
<tr>
<td>Total n-6 PUFA</td>
<td>23.27 ± 1.23^a</td>
<td>21.32 ± 0.60^a</td>
<td>12.62 ± 0.56^b</td>
<td>20.95 ± 0.59^a</td>
<td>13.43 ± 0.59^b</td>
</tr>
<tr>
<td>Total n-3 PUFA</td>
<td>1.52 ± 0.17^b</td>
<td>1.74 ± 0.10^b</td>
<td>10.58 ± 0.50^a</td>
<td>1.62 ± 0.14^b</td>
<td>11.29 ± 0.6^a</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>15.88 ± 1.81^a</td>
<td>12.44 ± 0.90^b</td>
<td>1.20 ± 0.05^c</td>
<td>13.26 ± 0.88^ab</td>
<td>1.20 ± 0.06^c</td>
</tr>
</tbody>
</table>

PUFA= polyunsaturated fatty acid, POS= positive control, NEG= negative control, FS= flaxseed, LD= low-dose estrogen therapy. LD= 13 µg, 90-day release. Different letters within a row denote statistical significance among groups at p<0.05 by one-way ANOVA followed by Student Newman Keul's post-hoc test. nd= not detected. Data are expressed as mean ± SEM. n=4-5/group.
microarchitecture in the spine. BMD and peak load of other skeletal sites, i.e. femur and tibia, were not responsive to FS and LD, either alone or in combination, suggesting that treatment-induced effects on BMD are site-specific. This study is also the first to report that FS, rich in ALA, alters fatty acid composition in the LV and tibia of ovariectomized rats.

Since the publication of the WHI trial (Anderson et al., 2004), the use of alternative pharmacological agents (e.g. bisphosphonates, selective ER modulators, parathyroid hormone) have become conventional for the prevention and treatment of menopausal-related bone-loss (Richman et al., 2006). However, due to the well-established benefits of estrogen therapy, formulations with lower doses of estrogen have become an attractive alternative to the standard dose formulations (Ettinger, 2007). LD offers advantages over standard-dose estrogen therapy in that it can effectively prevent and treat menopausal-related symptoms such as bone loss, albeit at a slightly lower percentage than that achieved using standard-dose estrogen therapy, without producing unacceptable side effects (Ettinger, 2007). LD increases BMD at the lumbar spine up to 5% in both healthy and osteopenic postmenopausal women (Lindsay et al., 2002; Gambacciani et al., 2008), and induces little or no benefit at the hip which is consistent with the results from our current study. We used a subcutaneous estrogen implant that models a transdermal delivery system which may also be associated with fewer side effects compared to standard-dose estrogen therapy (North American Menopause Society, 2010a).

Previous research has shown that combining long chain PUFA (e.g. EPA and DHA) with estrogen results in greater improvements in BMD and bone metabolism than either treatment alone (Schlemmer et al., 1999; Poulsen et al., 2008). Others have demonstrated that combining isoflavones with estrogen have no added benefit to bone mass and metabolism compared to estrogen alone (Cai et al., 2005). Our observation that LD resulted in BMD, structural integrity
and strength that were intermediate to the FS and FS+LD suggests an additive benefit to bone health when both components are combined. Thus, since LD is not quite as effective in preserving bone as standard doses, FS may offer extra protection against fractures to the LV when combined with LD.

Both the oil and lignan components of FS have been shown to modulate multiple mechanisms whereby estrogen preserves bone. For example, FS oil reduces serum tumor necrosis factor-α (Cohen et al., 2005) and ex-vivo release of PGE₂ from bone (Mollard et al., 2005). The mammalian lignans have also been shown to increase transcription of multiple markers of bone formation (Feng et al., 2008). However, the effect of FS alone or its components on bone metabolism has generally not demonstrated a clear benefit, which may in part be due to the fact that healthy women without low BMD have primarily been studied (Lucas et al., 2002; Brooks et al., 2004; Dodin et al., 2005). Thus, it is possible that the action on bone metabolism by ALA or its derivatives, or the mammalian lignans is apparent when osteoporosis is present or when FS or its components are combined with other compounds such as estrogen.

Another key finding from the present study is that FS consumption alters fatty acid composition of bones that differ in their ratio of cortical and trabecular bone. The total ALA content in the 10% FS diet used in this study is 2.36%, and the 10% FS level was chosen because it can be easily attained in the human diet, representing an intake of 25-50 g or 2½-5 tablespoons of FS per day. A 10% FS level has also been shown to modulate a number of biological indices of tumor growth, serum/plasma cholesterol, and atherosclerosis in animal and clinical models (Lucas et al., 2002; Dodin et al., 2005; Thompson et al., 2005; Bergman Jungestrom et al., 2007; Chen et al., 2007; Dupasquier et al., 2007; Lindahl et al., 2011). FS and FS+LD resulted in significantly higher relative levels of ALA and EPA in both the LV and tibia. Moreover, FS+LD
resulted in significantly higher ALA in the tibias and higher EPA in LV3 compared to FS alone. The reason for these findings are not yet clear but are suggested to involve estrogen. For example, less ALA undergoes β-oxidation in females compared to males (Childs et al., 2008). In addition, HRT use has been shown to increase plasma EPA and DHA or increase only plasma DHA in postmenopausal women (Sumino et al., 2003; Giltay et al., 2004). However, animal models have shown that estrogen (17β-estradiol) can decrease the activity of desaturase enzymes involved with the conversion of n-3 and n-6 PUFA into their longer-chain derivatives (Childs et al., 2008). Our observation that the POS control, NEG control and LD groups did not differ in their levels of the long-chain PUFA suggests that estrogen does not modulate fatty acid composition in the LV and tibias of ovariectomized rats. Nevertheless, it is possible that the combination of the lignans from FS along with LD may have modulated β-oxidation, the recycling of carbons or the conversion to longer-chain PUFA (Childs et al., 2008). It is important to note that when comparing the FS+LD group to the FS group, the EPA in the LV and ALA in the tibia were 0.25% and 0.84% higher, respectively, and the functional significance of these differences are not yet known. The direct effects of ALA on bone metabolism have not yet been elucidated while isolated EPA (>96% purity) has been shown to have biphasic effects, impairing bone loss or having no effect at low dietary levels, while accelerating bone loss at excessively high doses (1.0 g/kg body weight) (Poulsen & Kruger, 2006; Griel et al., 2007; Poulsen et al., 2008). The amount of EPA incorporated into the bone from the later study is unknown. Moreover, no significant relationships between fatty acids and BMD, BMC and peak load were observed suggesting that stronger bones due to the combination of FS and LD may be related to the interactive effects of FS components and LD or, to components of FS other than its fatty acid content such as the lignans.
The positive relationship observed between tibia BMC and femur BMC suggests that mineralization of long bones respond similarly to dietary and pharmacological treatments. A significant relationship however, was not observed when we correlated a more functional measure of bone strength, tibia peak load versus femur peak load. Thus, long bones may differ in their bone matrix composition. Because BMC in both the tibia and femur was positively and significantly associated with higher peak load at both these sites, these results suggest that mineral may be integrated at these different skeletal sites in a slightly diverse manner. Previous research have reported positive relationships between mineral and strength within the femur or tibia (Calero et al., 1996; Kubota et al., 1996), however, this is the first study to our knowledge that investigated the relationship of mineral and strength between the long bones. Thus, the significantly positive relationship between tibia BMC and femur BMC suggests that the mineral content of one long bone can be predictive of that in other long bones. This finding will require further validation by future studies, and whether this relationship is the same at other stages of the lifecycle needs to be confirmed.

In conclusion, FS combined with LD provides the greatest protection to BMD and bone structure compared to either treatment alone in ovariectomized rats. Interestingly, this benefit is site-specific to vertebrae with no benefit to long bones. This study is the first to demonstrate that FS, rich in ALA, results in the accumulation of n-3 PUFA such as ALA and EPA, and it results in diminished levels of n-6 PUFA such as linoleic acid and arachidonic acid in rat bones. These changes in fatty acid make biological sense as the changes in skeletal fatty acid composition mirror the dietary interventions. No significant relationships between fatty acid composition and BMD, BMC or peak load were observed suggesting that observed benefits to bone when FS is combined with LD are due to their interaction or that other components of FS may be responsible
for producing stronger bones when combined with LD. Whether the mammalian lignans are incorporated into the vertebrae and whether they along with ALA can modulate the action of estrogen on bone metabolism in postmenopausal osteoporosis are additional areas of future research.
5.0 Flaxseed does not antagonize the effect of ultra-low-dose estrogen therapy on bone mineral density and biomechanical bone strength in ovariectomized rats

5.1 Introduction

Evidence showing that lower dose estrogen therapies (Ettinger et al., 2004; Richman et al., 2006) are effective at inhibiting bone loss and have fewer side effects than their standard-dose counterparts has stimulated a renewed interest in HT for treating postmenopausal bone loss. As discussed in Section 2.3.2, estrogen therapy at low- and ultra-low doses reduce bone turnover and loss of BMD at the hip and LV, while reducing the likelihood of adverse side effects such as vaginal bleeding and endometrial hyperplasia (Ettinger et al., 2004; Gambacciani et al., 2008; Lindsay et al., 2002; Prestwood et al., 2003). Nevertheless, many women have begun seeking alternative or complementary therapies including dietary supplements such as FS. Many women view natural therapies as safer approaches in mitigating menopausal-related conditions than the currently available pharmacological options (Mahady et al., 2003; Grady, 2006).

FS is an oilseed rich in the n-3 PUFA, ALA; and the lignan, SDG. When consumed, the ALA in FS may be metabolized in small amounts into longer chain metabolites, EPA and DHA, compounds that were shown to inhibit bone loss (Sakaguchi et al., 1994; Watkins et al., 2006; Plourde & Cunnane, 2007). Similarly, SDG is metabolized into lignan metabolites, ED and EL (Thompson et al., 1991), compounds that were also found to modulate bone metabolism and be positively associated with BMD (Feng et al., 2008).

Because some women combine natural and pharmacological agents for disease prevention and treatment (Mahady et al., 2003; Singh & Levine, 2006) Study 1 (Chapter 4) investigated the effect of combining FS and LD estrogen therapy on BMD, bone structure and bone strength using the ovariectomized rat model of postmenopausal osteoporosis. This study
demonstrated that FS is neither beneficial nor detrimental to BMD, structure and strength but LD offers modest benefit to BMD, structure and strength at the LV. Of particular importance, was that the greatest benefit to the LV was observed when FS was combined with LD, suggesting that FS may offer extra protection against fractures to the LV when combined with LD. If combining FS with LD results in the strongest bones compared to either treatment alone, it was of interest to determine whether combining FS with even lower doses of estrogen (i.e. ULD) also attenuates bone loss or, whether this dose of estrogen is antagonized by a 10% FS diet since the lignans in FS may alter estrogen metabolism, bioavailability and action in the body (Power and Thompson, 2006). Thus, the objective of the present study was to compare the effect of FS combined with ULD versus ULD alone on bone mineral and bone strength in the ovariectomized rat model of postmenopausal osteoporosis. Since FS is one of the richest sources of n-3 PUFA and its oil is one of the top 10 most commonly used natural health products in Canada (Singh & Levine, 2006), a second objective was to determine whether the potential effect of FS+ULD on bone metabolism in ovariectomized rats is related to the modulation of bone tissue fatty acid composition.

5.2 Materials and Methods

5.2.1 Flaxseed Analysis

Protein, carbohydrate, fat, total fiber, lignan contents and fatty acid composition of the FS were analyzed as described in Chapter 4.

5.2.2 Animals and Diets

Animals and diets were as described in Chapter 4.
5.2.3 Experimental Design

Rats were acclimatized for one week on the BD after which they were randomized to the following groups (n=12/group): 1) negative (NEG) control fed the BD; 2) ULD, fed the BD and subcutaneously implanted with a 17β-estradiol pellet (7 µg, 90-day release; Innovative Research of America, Sarasota, FL, USA); and 3) FS+ULD, fed the FS diet and implanted with a 17β-estradiol pellet. A sham-operated group fed the BD was included as a POS group. The pellets were titrated to mimic ULD estrogen therapy (14 µg/day transdermal estrogen therapy) currently prescribed to postmenopausal women (North American Menopause Society, 2010b), and the pellets were placed subcutaneously in the interscapular region while rats were anesthesitized with isoflurane (3%) dissolved in oxygen with a flow rate of 1 liter per minute. The analgesic buprenorphine (0.05 mg/kg body weight) was administered subcutaneously following surgery. Measurements of food intake and body weights were performed daily and weekly, respectively. After 12-weeks of treatment, rats were sacrificed by CO2 asphyxiation followed by cervical dislocation and final body weight was determined. A twelve-week intervention, that approximates a one-year clinical trial in postmenopausal women, was chosen as the study duration (Baron et al., 1984; Kimmel et al., 1990; U.S. Food and Drug Administration, 1994). Femurs, tibias and LV1-3 were removed, cleaned of soft tissue, and stored at –80°C until further analyses. These skeletal sites were selected because they differ in their composition of trabecular and cortical bone. Trabecular bone, found in higher proportions at the LV, one of the most common sites of fragility fracture, is more metabolically active and more responsive to pharmacological and dietary intervention. Cortical bone, found in higher proportions in long bones such as the femur and tibia is less metabolically active (Morgan et al., 2008).
5.2.4 Bone Mineral Density and Bone Mineral Content of Lumbar Vertebrae, Femurs and Tibias

BMD and BMC of the LV1-3, whole and proximal 1/3 femurs and tibias were measured by DEXA as described in Chapter 4.

5.2.5 Biomechanical Strength Properties of Lumbar Vertebrae, Femurs and Tibias

Biomechanical strength properties of LV2, femurs and tibias were determined as described in Chapter 4.

5.2.6 Fatty Acid Composition of Lumbar Vertebrae and Tibia

Fatty acid composition of LV3 and right tibias were determined as described in Chapter 4.

5.2.7 Statistical Analyses

Differences between groups were determined by one-way ANOVA followed by the Student-Newman Keul’s post-hoc test (SigmaStat; Systat Software Inc, Version 3.5, Chicago, IL, USA).

A Kruskal-Wallis one-way ANOVA followed by the Dunn’s post-hoc test was used for data that were not normally distributed (LV1-3 BMC, LV2 peak load, femur BMD, upper 1/3 tibia BMD, LV3 ALA, tibia ALA, tibia EPA, tibia n-6/n-3 ratio). To determine the relationships between fatty acid composition and peak load in both the LV and tibias, linear regression analyses were performed on combined ovariectomized groups. Results are expressed as mean ± SEM. The significance level was set at $p < 0.05$.

5.3 Results

5.3.1 Flaxseed Lignan and Fatty Acid Composition

The proximate lignan and fatty acid composition of FS are shown in Chapter 4 (Table 4-1).
The total lignan concentration in the FS was 191.29 mg per 100 g of FS. Secoisolariciresinol was the primary lignan followed by lariciresinol, pinoresinol, and matairesinol.

Fatty acid composition is reported as a percentage of total fatty acids. ALA was the primary fatty acid detected in the FS sample (63.65% of total fatty acids), followed by linoleic, palmitic, stearic, and smaller amounts of oleic and cis-vaccenic acids (Table 4-1). No detectable levels of EPA or DHA were found in the FS sample.

5.3.2 Food Intake and Body Weight

The total food intake over the 12 week study was significantly lower in the POS group (1446 ± 30 g) compared to that in the ULD group (1583 ± 33 g) (p<0.05), while the total food intake in the NEG (1566 ± 37 g) and FS+ULD (1484 ± 41 g) groups did not differ from the POS and ULD groups. Moreover, the final body weight was significantly lower in the POS group (377 ± 13 g) compared to the NEG (443 ± 12 g), ULD (438 ± 14 g) and FS+ULD (415 ± 12 g) (p<0.05). No differences in % change of body weight from the beginning to the end of study were detected among any groups (data not shown).

5.3.3 Bone Mineral Density, Bone Mineral Content and Peak Load of Lumbar Vertebrae, Femurs and Tibias

LV1-3 BMD and LV2 peak load were significantly higher in the POS group compared to the NEG and ULD groups (p<0.05), while FS+ULD did not differ from POS, NEG and ULD groups (Table 5-1). No differences in LV1-3 BMC were found. In the whole femur, BMD was significantly higher in the POS group compared to all other groups (p<0.05) however, no other differences were observed among the ovariectomized groups. In the proximal region of the femur, BMD was significantly higher in the POS group compared to the NEG group (p<0.05) while ULD and the FS+ULD groups resulted in BMD that did not differ from the POS and NEG
Table 5-1: Bone mineral density (BMD), bone mineral content (BMC), and peak load in lumbar vertebrae, whole and regional femurs and tibias

<table>
<thead>
<tr>
<th></th>
<th>POS</th>
<th>NEG</th>
<th>ULD</th>
<th>FS+ ULD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumbar Vertebra</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LV1-3 BMD (mg/cm²)</td>
<td>203.79 ± 6.49a</td>
<td>172.37 ± 4.02b</td>
<td>178.87 ± 2.06b</td>
<td>183.45 ± 2.80ab</td>
</tr>
<tr>
<td>LV1-3 BMC (mg)</td>
<td>309.49 ± 13.51</td>
<td>271.77 ± 9.07</td>
<td>276.64 ± 2.99</td>
<td>290.97 ± 7.05</td>
</tr>
<tr>
<td>LV2 Peak Load (N)</td>
<td>419.82 ± 15.40a</td>
<td>353.97 ± 15.86b</td>
<td>362.65 ± 17.15b</td>
<td>396.61 ± 11.80ab</td>
</tr>
<tr>
<td>Whole Femur</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD (mg/cm²)</td>
<td>237.94 ± 6.43a</td>
<td>212.87 ± 2.84b</td>
<td>213.48 ± 1.69b</td>
<td>214.48 ± 3.83b</td>
</tr>
<tr>
<td>BMC (mg)</td>
<td>478.47 ± 19.31</td>
<td>455.71 ± 10.67</td>
<td>448.06 ± 5.74</td>
<td>459.15 ± 9.54</td>
</tr>
<tr>
<td>Midpoint Peak Load (N)</td>
<td>161.46 ± 4.28</td>
<td>169.87 ± 5.84</td>
<td>172.92 ± 3.77</td>
<td>177.09 ± 5.20</td>
</tr>
<tr>
<td>Proximal 1/3 Femur</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD (mg/cm²)</td>
<td>240.26 ± 7.18a</td>
<td>211.08 ± 3.90b</td>
<td>217.05 ± 1.51ab</td>
<td>216.12 ± 2.93ab</td>
</tr>
<tr>
<td>BMC (mg)</td>
<td>170.76 ± 7.33</td>
<td>159.04 ± 4.03</td>
<td>158.97 ± 2.19</td>
<td>161.58 ± 2.59</td>
</tr>
<tr>
<td>Whole Tibia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD (mg/cm²)</td>
<td>211.55 ± 3.73a</td>
<td>199.59 ± 2.34b</td>
<td>199.56 ± 1.04b</td>
<td>201.17 ± 3.74ab</td>
</tr>
<tr>
<td>BMC (mg)</td>
<td>362.98 ± 11.47</td>
<td>359.44 ± 8.61</td>
<td>353.42 ± 4.39</td>
<td>362.62 ± 7.61</td>
</tr>
<tr>
<td>Midpoint Peak Load (N)</td>
<td>112.91 ± 6.34</td>
<td>120.57 ± 6.66</td>
<td>122.63 ± 3.51</td>
<td>119.77 ± 3.97</td>
</tr>
<tr>
<td>Proximal 1/3 Tibia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMD (mg/cm²)</td>
<td>241.76 ± 5.63a</td>
<td>206.86 ± 1.70b</td>
<td>209.46 ± 1.86b</td>
<td>207.98 ± 4.69b</td>
</tr>
<tr>
<td>BMC (mg)</td>
<td>169.75 ± 6.358</td>
<td>153.20 ± 3.265</td>
<td>149.92 ± 1.786</td>
<td>152.98 ± 4.068</td>
</tr>
</tbody>
</table>

POS= positive control, NEG= negative control, FS= flaxseed, ULD= ultra-low-dose estrogen therapy. ULD= 7 µg, 90-day release. Different letters within a row denote statistical significance among groups at p<0.05 by one-way ANOVA followed by Student Newman Keul's post-hoc test. Data are expressed as mean ± SEM. n=10-12/group.
groups. No differences in BMC or midpoint peak load were observed in the whole or proximal femur (Table 5-1). In the whole tibia, BMD was significantly higher in the POS group compared to the NEG and ULD groups (p<0.05) while FS+ULD did not differ from POS, NEG and ULD groups. No differences in BMC or midpoint peak load were observed in the whole tibia. In the proximal region of the tibia, BMD was significantly higher in the POS group compared to the other groups (p<0.05) however, no differences were observed among the ovariectomized groups. No differences in BMC at the proximal region of the tibia were found.

5.3.4 Fatty Acid Composition of Lumbar Vertebrae and Tibias

In the LV3, relative levels of ALA and its long-chain metabolite, EPA, were significantly higher in the FS+ULD group compared to all other groups (p<0.001) (Table 5-2). DHA was also significantly higher in the FS+ULD group compared to the NEG and ULD groups (p<0.05) but did not differ from the POS group. The relative levels of linoleic acid were significantly lower in the FS+ULD group compared to all groups (p<0.001) while no significant differences in levels of its long-chain metabolite, arachidonic acid were observed. As expected, total n-3 PUFA was significantly higher in the FS+ULD group (p<0.001) while the total n-6 PUFA levels and the n-6/n-3 ratio were significantly lower in the FS+ULD group compared to all other groups (p<0.001). Total polyunsaturates were significantly higher in the FS+ULD group compared to all groups (p<0.05), while no differences in monounsaturates were observed. Total saturates were significantly lower in the FS+ULD and ULD groups compared to the POS group (p<0.05).

Similar to LV3, relative levels of ALA and EPA in the tibia were significantly higher in the FS+ULD group compared to all other groups (p<0.001) (Table 5-3). Linoleic acid and arachidonic acid levels were significantly lower in the FS+ULD group compared to all other groups (p<0.001 for linoleic acid, p<0.05 for arachidonic acid). Total n-3 PUFA was
### Table 5-2: Fatty acid composition of lumbar vertebra 3 (LV3) expressed as percentage of total fatty acids

<table>
<thead>
<tr>
<th>POS</th>
<th>NEG</th>
<th>ULD</th>
<th>FS+ULD</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0 (myristic)</td>
<td>1.62 ± 0.08</td>
<td>2.05 ± 0.08</td>
<td>1.86 ± 0.22</td>
</tr>
<tr>
<td>C14:1 n-5 (myristoleic)</td>
<td>0.33 ± 0.08</td>
<td>0.33 ± 0.04</td>
<td>0.32 ± 0.04</td>
</tr>
<tr>
<td>C16:0 (palmitic)</td>
<td>25.64 ± 0.25a</td>
<td>26.63 ± 0.44a</td>
<td>25.98 ± 0.40a</td>
</tr>
<tr>
<td>C16:1 n-7 (palmitoleic)</td>
<td>5.90 ± 0.58b</td>
<td>8.63 ± 0.53b</td>
<td>10.01 ± 0.62a</td>
</tr>
<tr>
<td>C18:0 (stearic)</td>
<td>9.94 ± 1.50a</td>
<td>6.18 ± 0.25b</td>
<td>5.58 ± 0.30b</td>
</tr>
<tr>
<td>C18:1 n9 (oleic)</td>
<td>27.78 ± 1.50</td>
<td>28.34 ± 0.41</td>
<td>29.58 ± 0.65</td>
</tr>
<tr>
<td>C18:1 n7 (cis-vaccenic)</td>
<td>3.48 ± 0.16ab</td>
<td>3.79 ± 0.07a</td>
<td>3.82 ± 0.13a</td>
</tr>
<tr>
<td>C18:2 n6 (linoleic)</td>
<td>15.12 ± 0.61a</td>
<td>15.92 ± 0.70a</td>
<td>14.48 ± 0.37a</td>
</tr>
<tr>
<td>C18:3 n6 (γ-linolenic)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C18:3 n3 (α-linolenic)</td>
<td>0.95 ± 0.03b</td>
<td>1.23 ± 0.04b</td>
<td>1.21 ± 0.02b</td>
</tr>
<tr>
<td>C20:0 (arachidic)</td>
<td>0.30 ± 0.11</td>
<td>0.12 ± 0.01</td>
<td>0.10 ± 0.04</td>
</tr>
<tr>
<td>C20:2 n6 (eicosadienoic)</td>
<td>0.37 ± 0.04</td>
<td>0.41 ± 0.01</td>
<td>0.39 ± 0.02</td>
</tr>
<tr>
<td>C20:3 n6 (eicosatrienoic)</td>
<td>0.44 ± 0.10</td>
<td>0.39 ± 0.02</td>
<td>0.36 ± 0.04</td>
</tr>
<tr>
<td>C20:3 n3 (eicosatrienoic)</td>
<td>0.14 ± 0.07</td>
<td>0.08 ± 0.03</td>
<td>0.39 ± 0.30</td>
</tr>
<tr>
<td>C20:4 n6 (arachidonic)</td>
<td>5.21 ± 0.48</td>
<td>3.90 ± 0.35</td>
<td>3.82 ± 0.26</td>
</tr>
<tr>
<td>C20:5 n3 (eicosapentaenoic)</td>
<td>0.22 ± 0.08b</td>
<td>0.44 ± 0.10b</td>
<td>0.38 ± 0.07b</td>
</tr>
<tr>
<td>C22:0 (behenic)</td>
<td>0.25 ± 0.11</td>
<td>0.04 ± 0.03</td>
<td>0.04 ± 0.04</td>
</tr>
<tr>
<td>C22:1 n9 (erucic)</td>
<td>0.05 ± 0.03</td>
<td>nd</td>
<td>0.02 ± 0.02</td>
</tr>
<tr>
<td>C22:2 n6 (docosadienoic)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C22:4 n6 (adrenic)</td>
<td>0.93 ± 0.19a</td>
<td>0.53 ± 0.06ab</td>
<td>0.66 ± 0.15ab</td>
</tr>
<tr>
<td>C22:5 n6 (docosapentenoic)</td>
<td>0.12 ± 0.05</td>
<td>0.08 ± 0.03</td>
<td>0.11 ± 0.04</td>
</tr>
<tr>
<td>C22:5 n3 (docosapentaenoic)</td>
<td>0.63 ± 0.02b</td>
<td>0.41 ± 0.04b</td>
<td>0.39 ± 0.02b</td>
</tr>
<tr>
<td>C22:6 n3 (docosahexaenoic)</td>
<td>0.86 ± 0.09ab</td>
<td>0.51 ± 0.05b</td>
<td>0.50 ± 0.05b</td>
</tr>
</tbody>
</table>
### Table 5-2 (continued): Fatty acid composition of lumbar vertebra 3 (LV3) expressed as percentage of total fatty acids

<table>
<thead>
<tr>
<th></th>
<th>POS</th>
<th>NEG</th>
<th>ULD</th>
<th>FS+ULD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Saturates</td>
<td>37.74 ± 1.78a</td>
<td>35.02 ± 0.61ab</td>
<td>33.55 ± 0.52b</td>
<td>33.20 ± 0.81b</td>
</tr>
<tr>
<td>Total Monounsaturates</td>
<td>37.54 ± 1.96</td>
<td>41.08 ± 0.77</td>
<td>43.76 ± 0.98</td>
<td>38.72 ± 2.32</td>
</tr>
<tr>
<td>Total Polyunsaturates</td>
<td>24.72 ± 0.47b</td>
<td>23.89 ± 0.75b</td>
<td>22.69 ± 0.94b</td>
<td>28.08 ± 1.67a</td>
</tr>
<tr>
<td>Total n-6 PUFA</td>
<td>22.19 ± 0.34a</td>
<td>21.23 ± 0.77a</td>
<td>19.83 ± 0.66a</td>
<td>14.96 ± 1.38b</td>
</tr>
<tr>
<td>Total n-3 PUFA</td>
<td>2.52 ± 1.18b</td>
<td>2.66 ± 0.09b</td>
<td>2.86 ± 0.38b</td>
<td>13.13 ± 0.37a</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>8.97 ± 0.62a</td>
<td>8.04 ± 0.45a</td>
<td>7.30 ± 0.61a</td>
<td>1.13 ± 0.08b</td>
</tr>
</tbody>
</table>

POS= positive control, NEG= negative control, FS= flaxseed, ULD= ultra-low-dose estrogen therapy. ULD= 7 µg, 90-day release. Different letters within a row denote statistical significance among groups at p<0.05 by one-way ANOVA followed by Student Newman Keul's post-hoc test, or by a Kruskal-Wallis one-way ANOVA followed by the Dunn’s post-hoc test for data that was not normally distributed. nd= not detected. Data are expressed as mean ± SEM. n=5-6/group.
Table 5-3: Fatty acid composition of tibia expressed as percentage of total fatty acids

<table>
<thead>
<tr>
<th></th>
<th>POS</th>
<th>NEG</th>
<th>ULD</th>
<th>FS+ULD</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14:0 (myristic)</td>
<td>1.46 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.94 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.07 ± 0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.15 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C14:1 n-5 (myristoleic)</td>
<td>0.23 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.38 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.39 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.41 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C16:0 (palmitic)</td>
<td>21.45 ± 0.44&lt;sup&gt;b&lt;/sup&gt;</td>
<td>23.01 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.01 ± 0.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22.73 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C16:1 n-7 (palmitoleic)</td>
<td>9.34 ± 0.79&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.01 ± 0.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.58 ± 0.79&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.34 ± 0.32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:0 (stearic)</td>
<td>4.62 ± 0.27&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.65 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.46 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.73 ± 0.14&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:1 n9 (oleic)</td>
<td>34.02 ± 1.40</td>
<td>31.85 ± 0.42</td>
<td>32.72 ± 0.64</td>
<td>31.46 ± 0.40</td>
</tr>
<tr>
<td>C18:1 n7 (cis-vaccenic)</td>
<td>4.09 ± 0.14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.09 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.03 ± 0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.48 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:2 n6 (linoleic)</td>
<td>19.46 ± 1.69&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.81 ± 0.64&lt;sup&gt;a&lt;/sup&gt;</td>
<td>17.05 ± 0.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.96 ± 0.27&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:3 n6 (γ-linolenic)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C18:3 n3 (α-linolenic)</td>
<td>1.06 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.30 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.21 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.65 ± 0.29&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C18:4 n3 (stearidonic)</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.14 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:0 (arachidic)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C20:2 n6 (eicosadienoic)</td>
<td>0.29 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.33 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.31 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.20 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:3 n6 (eicosatrienoic)</td>
<td>0.09 ± 0.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.21 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.16 ± 0.02&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.13 ± 0.01&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:3 n3 (eicosatrienoic)</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.20 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:4 n6 (arachidonic)</td>
<td>2.92 ± 0.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.55 ± 0.20&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>2.20 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.48 ± 0.10&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>C20:5 n3 (eicosapentaenoic)</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.06 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.78 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C22:0 (behenic)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C22:1 n9 (erucic)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C22:2 n6 (docosadienoic)</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C22:4 n6 (adrenic)</td>
<td>0.47 ± 0.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.39 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.34 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.10 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>C22:5 n6 (docosapentaenoic)</td>
<td>0.04 ± 0.04</td>
<td>0.03 ± 0.02</td>
<td>0.02 ± 0.02</td>
<td>nd</td>
</tr>
<tr>
<td>C22:5 n3 (docosapentaenoic)</td>
<td>0.15 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.27 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.20 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.86 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C22:6 n3 (docosahexaenoic)</td>
<td>0.31 ± 0.11</td>
<td>0.12 ± 0.08</td>
<td>0.26 ± 0.07</td>
<td>0.20 ± 0.12</td>
</tr>
</tbody>
</table>
Table 5-3 (continued): Fatty acid composition of tibia expressed as percentage of total fatty acids

<table>
<thead>
<tr>
<th></th>
<th>POS</th>
<th>NEG</th>
<th>ULD</th>
<th>FS+ULD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Saturates</td>
<td>27.54 ± 0.70</td>
<td>28.59 ± 0.24</td>
<td>28.54 ± 0.46</td>
<td>28.61 ± 0.34</td>
</tr>
<tr>
<td>Total Monounsaturates</td>
<td>47.68 ± 1.51</td>
<td>48.34 ± 0.62</td>
<td>49.72 ± 0.83</td>
<td>47.69 ± 0.19</td>
</tr>
<tr>
<td>Total Polyunsaturates</td>
<td>24.79 ± 1.32</td>
<td>23.06 ± 0.59</td>
<td>21.74 ± 1.06</td>
<td>23.69 ± 0.34</td>
</tr>
<tr>
<td>Total n-6 PUFA</td>
<td>23.27 ± 1.23&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.32 ± 0.60&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>20.08 ± 1.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>12.85 ± 0.26&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total n-3 PUFA</td>
<td>1.52 ± 0.17&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.74 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.66 ± 0.08&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.84 ± 0.045&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>n-6/n-3</td>
<td>15.88 ± 1.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.44 ± 0.90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>12.18 ± 0.77&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.20 ± 0.07&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

POS= positive control, NEG= negative control, FS= flaxseed, ULD= ultra-low-dose estrogen therapy. ULD= 7 µg, 90-day release. Different letters within a row denote statistical significance among groups at p<0.05 by one-way ANOVA followed by Student Newman Keul's post-hoc test, or by a Kruskal-Wallis one-way ANOVA followed by the Dunn’s post-hoc test for data that was not normally distributed. nd= not detected. Data are expressed as mean ± SEM. n=5-6/group.
significantly higher in the FS+ULD group (p<0.001) while the total n-6 PUFA and the n-6/n-3 ratio were significantly lower compared to all other groups (p<0.001). No differences in tibia DHA, total polyunsaturates, monounsaturates or saturates were observed.

### 5.3.5 Relationships Between Fatty Acid Composition and Bone Outcome Measures

Linear regressions were performed between PUFA (linoleic acid, ALA, arachidonic acid, EPA, DHA, total n-3 PUFA, total n-6 PUFA, and n-6/n-3 ratio) and BMD, BMC and peak load at both the LV and tibia. In LV3, relative levels of ALA and total n-3 PUFA were significantly and positively related with LV2 peak load \[
\begin{align*}
  r &= 0.52, \text{for LV2 Peak Load=350} + (5 \times \text{LV3 ALA}); \\
  r &= 0.52, \text{LV2 Peak Load=345} + (4 \times \text{LV3 Total n-3})
\end{align*}
\]
Moreover, linoleic acid and total n-6 PUFA were significantly and inversely related with LV1-3 BMC \[
\begin{align*}
  r &= -0.62, \text{LV1-3 BMC= 335 – (4 \times \text{LV3 linoleic acid})}; \\
  r &= -0.50, \text{LV1-3 BMC= 324 – (3 \times \text{LV3 Total n-6})}, \text{while n-6/n-3 ratio was significantly and inversely related with LV2 peak load \[r = -0.48, \text{LV2 Peak Load= 396 – (5 \times \text{LV3 n-6/n-3})}\] and LV1-3 BMD \[r = -0.51, \text{LV1-3 BMD= 186 – (2 \times \text{LV3 n-6/n-3})}\]. In the tibia, DHA was significantly and inversely related to BMD \[r = -0.68, \text{BMD= 203 – (17\times \text{tibia DHA})}\]. No other significant correlations were observed between skeletal fatty acid composition and bone outcome measures.

### 5.4 Discussion

Our data demonstrate that FS+ULD attenuates the loss of LV1-3 BMD and LV2 peak load, as well as tibia BMD. The fact that ULD and FS+ULD treatments did not significantly differ from each other in any outcomes suggests that FS does not antagonize the effect of ULD on bone. In contrast to the tibia, no treatment-induced effects on BMD, BMC or strength were observed in the whole femur- another skeletal site rich in cortical bone- suggesting that
FS+ULD induces skeletal-specific effects. The observation that both ULD and FS+ULD attenuated BMD at the proximal region of the femur suggests that trabecular bone is more responsive than cortical bone to treatments, consistent with previous research (Breitman et al., 2003, 2005). Moreover, FS does not antagonize the effect of ULD in this trabecular-rich region of the skeleton. It is important to note however that FS+ULD attenuated the loss of BMD in the whole tibia while no treatment-induced effects were observed at the proximal region of this bone suggesting that the benefits of ULD and FS+ULD on trabecular bone are site-specific.

While Study 1 (Chapter 4) (Sacco et al., 2009b) demonstrated for the first time that FS combined with LD estrogen therapy results in a significant benefit to BMD, bone structure and bone strength at the LV compared to NEG alone, the current combination of the ULD and FS had a weaker effect, such that bone mineral and strength at the LV and proximal region of the femur did not differ from the POS and NEG groups. These contrasting findings between studies are likely due to the lower dose of estrogen - a 54% lower dose of estrogen – used in the current study. Our observations that FS+ULD did not result in higher BMD in the whole femur or greater peak load at the femur and tibia midpoints compared to the NEG group also indicate that FS+ULD induce site-specific effects in skeletal areas richer in trabecular bone. Due to these weak effects, we decided not to analyze bone structure.

It is possible that the metabolites resulting from FS ingestion exert estrogen- or anti-inflammatory-like activity, protecting against ovariectomy-induced bone loss. For example, 7 days of incubation with doses as low as 0.01 mg/mL of ED and EL increased mRNA expression of osteonectin and type I collagen in the MG-63 human osteosarcoma cell line which is a human osteoblast-like cell line (Feng et al., 2008). Human and animal data showed that FS oil or a high
ALA diet can result in lower levels of serum tumor necrosis factor-α and PGE₂ in bone (Mollard et al., 2005; Griel et al., 2007).

FS combined with ULD resulted in higher n-3 PUFA and lower n-6 PUFA in both the LV3 and tibia, in agreement with Study 1 (Chapter 4) (Sacco et al., 2009b). ULD did not alter bone fatty acid composition suggesting, as expected, that the observed effects are due to FS alone. There was a significant and positive relationship between total n-3 PUFA and peak load at the LV, as well as ALA and peak load at the LV indicating that the relationship between total n-3 PUFA and peak load are primarily due to ALA and not other n-3 PUFA such as EPA. The beneficial effects of a high ALA diet, in which FS oil was a predominant source of ALA, on bone health has been reported in adult men and women. This study demonstrated that a diet high in ALA resulted in significantly lower serum levels of NTX, a marker of bone resorption, compared to the high linoleic acid diet (Griel et al., 2007). Nonetheless, it is not known whether the effect of the high ALA diet on markers of bone metabolism in this study by Griel et al. (2007) were due to ALA or its long-chain metabolites. It is prudent to note that the significant relationships between fatty acids and bone outcome measures do not infer a cause and effect.

Since LV3 ALA and total n-3 PUFA content were not related to LV1-3 BMC, this suggests that the n-3 PUFA modulate factors of bone quality (e.g. matrix composition, microarchitecture of cancellous bone) to enhance bone health at the lumbar spine. In contrast, the inverse relationships between LV3 linoleic acid and LV1-3 BMC; LV3 total n-6 PUFA and LV1-3 BMC; and LV3 n-6/n-3 ratio with LV2 peak load and LV1-3 BMD suggests that the n-6 PUFA may modulate both quantity (i.e. mineral content) and quality (i.e. matrix composition, microarchitecture) of the LV. Unexpectedly, a significant inverse relationship was observed between DHA and BMD in the tibia. DHA was previously shown to inhibit bone loss in
ovariectomized rats (Watkins et al., 2006) and the reasons for the discrepancy between our results with those from previous literature may be due to differences in DHA levels that are incorporated into bone, however, this warrants further investigation. Nonetheless, the differences in DHA levels observed in the current study are less than 0.20% and did not reach statistical significance. Thus, further investigation is necessary to determine the potential biological role of DHA at these levels on bone metabolism in the current study design.

Ovariectomy-induced hyperphagia and subsequent weight gain has been extensively documented in the literature (Jiang et al., 2008; McElroy & Wade, 1987; Roy & Wade, 1977). The present study has also demonstrated higher final body weights among all ovariectomized groups (NEG, ULD, FS+ULD) that can be attributed to higher food intakes. However, a recent study by our laboratory has demonstrated that higher food intake and weight gain post-ovariectomy do not modulate BMD or strength at the femur or LV (Jiang et al., 2008).

In conclusion, data from the current study demonstrated that ULD therapy, at a clinically relevant dose, when combined with FS, attenuates the deterioration of bone tissue at the LV. Moreover, FS does not antagonize the activity of ULD when given in combination. Whether a similar effect is observed in postmenopausal women who are seeking complimentary therapies for postmenopausal osteoporosis should be determined.
CHAPTER 6

Study 3

6.0 Accessibility of $^3$H-secoisolariciresinol diglycoside lignan metabolites in skeletal tissue of ovariectomized rats.

6.1 Introduction

Dietary estrogens (i.e. phytoestrogens) such as isoflavones from soy and lignans from FS are currently being investigated as a natural strategy to manage menopausal-relating conditions. Study 1 (Chapter 4) (Sacco et al., 2009b) showed, using the established ovariectomized rat model of postmenopausal osteoporosis, that 10% dietary FS, rich in the phytoestrogen lignan SDG, is neither protective nor detrimental to BMD, bone structure or bone strength. Interestingly, a synergistic effect on BMD, structure and strength at the LV was observed when FS was combined with LD. These findings are relevant to postmenopausal women, particularly those concerned with preserving bone health as there is no ideal treatment for postmenopausal loss of BMD. Moreover, Western women who take prescription drugs such as HT also commonly consume dietary supplements, many of which contain phytoestrogens (Mahady et al., 2003; Singh & Levine, 2006). While estrogen therapy is not a main-line treatment for managing bone health, its usefulness in younger postmenopausal women is being re-evaluated and, it may be prescribed in the short-term to help alleviate hot flushes, night sweats and to prevent bone loss that is associated with early menopause (Pines, 2009; Lau et al., 2010). In addition, the number of food products containing FS has tripled since 2003 (Fitzpatrick, 2007). Examples of foods in which FS has been introduced include breads, cereals, cereal bars, pasta and yogurt. SDG is also found in appreciable amounts in vegetables, fruits, whole grains and berries (Mazur, 1998; Thompson et al., 2006). Consequently, the consumption of FS and SDG is widespread in the Western diet.
The mechanisms whereby the combination of FS and LD exert their greatest protection to bone mass and bone strength at the LV are currently unknown but may be related to the rich levels of lignan SDG and n-3 PUFA, ALA. While Study 1 (Chapter 4) (Sacco et al., 2009b) showed that feeding a 10% FS diet to ovariectomized rats results in higher proportions of n-3 PUFA including ALA and its longer-chain metabolite ALA in LV and tibias, it has not yet been shown whether SDG metabolites (secoisolariciresinol, ED, EL and other minor metabolites) are accessible to skeletal tissue. If so, lignans may alter the action of LD through direct action on bone cells. Previous studies have reported the deposition of SDG metabolites into various organs (Rickard & Thompson, 1998; Saarinen et al., 2008; Saarinen & Thompson, 2010) but not into the skeleton. Thus, the objective of this study was to determine, using the ovariectomized rat model of postmenopausal osteoporosis and radiolabeled SDG, whether SDG metabolites are accessible to the skeleton. Since the distribution of SDG metabolites varies among tissues (Rickard & Thompson, 1998; Saarinen et al., 2008; Saarinen & Thompson, 2010), it is not known how the accessibility of SDG metabolites in skeletal tissue compares to that in other tissues. Thus, a secondary objective of this study was to compare the radioactive levels of SDG metabolites in skeletal tissue with that in other tissues (i.e. liver, uterus, vagina, mammary gland, thymus, spleen, kidney, lungs, bladder, heart and gastrointestinal tissue). By determining whether SDG metabolites are accessible to skeletal tissue, this study will provide insight into whether it is possible that lignans can have a direct role in bone metabolism when FS is combined with LD.
6.2 Materials and Methods

6.2.1 Radiolabeling of Secoisolariciresinol Diglycoside

Purified SDG was prepared as previously described (Rickard et al., 1996). The benzyl methylenes of the purified SDG were then labeled with tritium using a gas exchange method by Amersham International (Little Chalfont, Buckinghamshire, UK) (Rickard & Thompson, 1998), resulting in specific radioactivity of 999 GBq/mol and a radiochemical purity of 98.5%. The benzyl methylene groups were chosen as the labeling site because tritium is more stable at the benzyl methylene groups in comparison to the benzene ring of SDG (Rickard & Thompson, 1998; Rickard & Thompson, 2000). Moreover, the benzyl methylene groups remain intact during metabolism of SDG by colonic microflora to its metabolites, ED and EL (Rickard & Thompson, 2000).

6.2.2 Animals, Diets and Experimental Design

Three-month old ovariectomized Sprague Dawley rats (n=6, mean body weight 207 ± 3 g) were purchased from Charles River Laboratories (St-Constant, QC, Canada), housed one per cage and maintained at 22°–24°C and 50% humidity with 12:12-hr light:dark cycle. Rats were acclimatized to the basal diet (BD), an AIN-93M (Reeves et al., 1993) formulation (Table 6-1), for the first seven days. After this seven day period, rats were switched to the FS diet which was the BD supplemented with 10% freshly ground FS (Johnson Seeds Ltd, Arborg, MB, Canada) (Table 6-1) and received a daily 500 µl gavage of tritium-labeled SDG ($^3$H-SDG) (7.4 kBq/g body weight) in deionized water (n=3), or deionized water alone (n=3). The dose of $^3$H-SDG represents a level that can be detected in rodent tissues and a level in which no previous harmful effects have been observed (Saarinen et al., 2008). This dose of $^3$H-SDG also contributes a negligible amount of SDG (<1%) to the whole FS diet (Rickard & Thompson, 1998). The dose
Table 6-1: Composition of basal diet (AIN-93M) and 10% flaxseed (FS) diet

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Basal Diet (AIN-93M)</th>
<th>10% FS Diet*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g/kg</td>
<td>kcal/kg</td>
</tr>
<tr>
<td>Casein</td>
<td>140</td>
<td>501.2</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>465.69</td>
<td>1676.49</td>
</tr>
<tr>
<td>Dyetrose</td>
<td>155</td>
<td>589</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
<td>400</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>Soybean Oil</td>
<td>40</td>
<td>360</td>
</tr>
<tr>
<td>t-Butylhydroquinone</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>Salt Mix (# 210050)</td>
<td>35</td>
<td>30.8</td>
</tr>
<tr>
<td>Vitamin Mix (# 310025)</td>
<td>10</td>
<td>38.7</td>
</tr>
<tr>
<td>L-Cysteine</td>
<td>1.8</td>
<td>7.2</td>
</tr>
<tr>
<td>FS</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Choline Bitartrate</td>
<td>2.5</td>
<td>0</td>
</tr>
</tbody>
</table>

Total                  | 1000     | 3603.39   | 1000     | 3600.08   |

*Secoisolariciresinol content, 178.73 ± 62.06 mg/100g FS, α-linolenic acid (ALA) content, 23.55 ± 0.74 g/100g FS (Study 1, Chapter 4) (Sacco et al., 2009b).
of FS chosen for this research is a level that can be attained in the human diet (25-50 g/day) as whole or ground seed incorporated into foods such as muffins and cereals (Nesbitt et al., 1999; Brooks et al., 2004; Thompson et al., 2005). At the end of the 7 day intervention, rats were killed 12 hours following the last oral gavage, and skeletal samples (LV, femur and tibia), non-skeletal samples (serum, liver, kidney, bladder, spleen, brain, heart, mammary, uterus, vagina, thymus, lungs) including gastrointestinal tissues (stomach, small intestines, cecum and colon) were dissected, rinsed with phosphate buffered saline, and frozen at -80°C for later analyses. The time point chosen for sacrifice was based on previous research that showed high levels of radioactivity in tissue and blood at this time post-dose (Rickard & Thompson, 1998). Animal care and use were carried out in accordance with the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care, 1993), and the experimental protocol was approved by the Animal Ethics Committee at the University of Toronto, Toronto, Canada.

6.2.3 Determination of Radioactivity in Tissues and Serum

Determination of radioactivity in tissues and serum was conducted in both the 3H-SDG and control groups. For scintillation counting of skeletal tissue (n=3 per skeletal site), bone marrow was flushed from femurs and tibias with phosphate buffered saline. Lumbar vertebrae (200-280 mg), femurs (325-600 mg) and tibias (325-500 mg) were subsequently rinsed three times with phosphate buffered saline to remove residual blood. Individual bones were dipped in liquid nitrogen, crushed with a hammer, and large bone masses were then pulverized by mortar and pestle under liquid nitrogen as previously described (Study 1, Chapter 4) (Sacco et al., 2009b).

Individual samples were then dissolved in a mixture of 1M hyamine hydroxide in methanol (Packard Bioscience B.V., Groningen, Netherlands) and deionized water (9:1 v/v), and were incubated overnight in a shaking water bath at 45°C. 15 mL of scintillant (Cytoscint ES, ICN,
Biomedicals, Costa Mesa, CA, USA) was then added to the dissolved tissue samples and the samples were mixed vigorously. To minimize chemiluminescence, 0.5 mL of 0.5 M glacial acetic acid was added to each vial. Vials were then shaken and allowed to equilibrate in the dark for 4 days before scintillation counting.

For scintillation counting of non-skeletal tissue, samples were processed according to the methods described earlier (Rickard & Thompson, 1998; Saarinen et al., 2008; Saarinen & Thompson, 2010). Samples (80-160 mg) were minced and dissolved in a mixture of 1M hyamine hydroxide in methanol (Packard Bioscience B.V., Groningen, Netherlands) and deionized water (9:1 v/v), and were incubated overnight in a shaking water bath at 45°C. Dissolved samples (100 μL) were then mixed with 5 mL scintillant. For tissues with residual blood, 100 μL of 9.7 M hydrogen peroxide was added followed by heating the samples at 55-60°C for 30 minutes with the vials loosely capped. Once cooled, 0.5 mL of 0.5 M glacial acetic acid was added to the samples to minimize chemiluminescence and the vials were vigorously mixed before being stored in the dark for 4 days before scintillation counting.

For scintillation counting of serum, 50 μL of serum was dissolved in 300 μL of hyamine hydroxide (Packard Bioscience B.V., Groningen, Netherlands) in ethanol (1:2, v/v). Samples were then incubated in a shaking water bath at 55-60°C for 1 hour after which 0.5 mL of 9.7 M hydrogen peroxide was added to prevent color quenching. Vials were incubated for another 30 min at 55-60°C then cooled before adding scintillant and glacial acetic acid to minimize chemiluminescence. Vials were then mixed vigorously and stored in the dark for 4 days before scintillation counting (Rickard & Thompson, 1998).

All samples were counted for 5-min periods using the TRI-CARB 2900 TR Liquid Scintillation analyzer (Packard Instrument Co., Meriden, CT, USA) with a mean counting
efficiency of 63.34% for tritium based on unquenched controls. Corrections for chemical and color quenching of the samples were done automatically by the liquid scintillation counter. All results were corrected for tissue-specific counting efficiencies and possible losses during processing as previously described (Rickard & Thompson, 1998). All results were converted to picomole equivalents of $^3$H-SDG by dividing them by the specific activity of the radioisotope (27 mCi/mL) and were adjusted for the weight or volume of tissues and serum.

6.2.4 Statistical Analyses

To detect differences in radioactivity within a particular tissue between the control and $^3$H-SDG groups, an unpaired Student’s t-test was performed. Differences in radioactivity among various tissues within a group (i.e. control, or $^3$H-SDG treated rats) were determined by one-way analysis of variance (ANOVA) followed by a Student-Newman-Keul’s test as the post-hoc test for data that was normally distributed and had equal variances. For data that was not normally distributed (gastrointestinal tissues) or did not have equal variances (non-skeletal tissue), a Kruskal-Wallis one-way ANOVA followed by the Dunn’s post-hoc test was performed. All statistics were performed using SigmaStat (Systat Software Inc, Version 3.5, Chicago, IL, USA). The significance level was set at $p<0.05$. All data are presented as mean radioactivity per gram of tissue weight ± standard error of the mean.

6.3 Results

6.3.1 Final Body and Organ Weights

There were no significant differences in body weight between the control and $^3$H-SDG groups at either the beginning (control: 206 ± 7 g, $^3$H-SDG: 207 ± 3 g) or end (control: 215 ± 10 g, $^3$H-
SDG: 216 ± 4 g) of the one-week study. Final tissue weights were similar between the control and $^{3}$H-SDG groups (data not shown).

6.3.2 Radioactivity in Tissues and Serum

Administration of $^{3}$H-SDG resulted in detectable radioactivity in all collected tissues (Figure 6-1A). Radioactivity in gastrointestinal tissues (i.e. cecum, colon, stomach and small intestine) represented 65.2% of the total radioactivity detected, which was primarily due to concentrations of radioactivity in the cecum and colon (Figure 6-1A).

In non-gastrointestinal tissues, kidney had highest level of radioactivity (4.5% of the total measured radioactivity), while skeletal tissue levels had the lowest (0.1% of total radioactivity) (Figure 6-1A). In skeletal tissue, radioactivity in the LV, tibia and femur represented 44.7%, 32.2% and 23.1% of the total radioactivity measured in the skeleton, respectively (Figure 6-1B). Overall, the radioactivity concentrations from highest to lowest were:

cecum>colon>kidney>thymus>brain>heart>spleen>liver>lung>stomach>bladder>vagina>small intestine>uterus>mammary>LV>tibia>femur. As expected, no radioactivity was detected in the tissues of control rats.

Radioactivity in the LV was 1.4 to 1.9 times higher than the tibia and femur, respectively, but they did not differ significantly (Figure 6-2A). Radioactivity in the total skeletal tissue was significantly lower than the thymus, heart, brain, liver and kidney ($p<0.001$) (Figure 6-2B). No differences in radioactivity were observed between total skeletal tissue and mammary, uterus, vagina, spleen, bladder and lung tissues (Figure 6-2B). Excluding skeletal tissue, the highest level of radioactivity was observed in the kidney, which was 1.1 to 3.5 times higher than all other non-gastrointestinal tissues. Radioactivity in the kidney was significantly higher compared to mammary tissue ($p<0.001$), but it did not differ significantly from other non-gastrointestinal
Figure 6-1: Percent distribution of $^3$H-secoisolariciresinol diglycoside ($^3$H-SDG) metabolites in (A) total collected tissues and (B) skeletal tissues of ovariectomized rats. n=3 rats.
Figure 6-2: Mean levels of radioactivity in (A) skeletal tissues, (B) non-gastrointestinal tissues, and (C) gastrointestinal tissues in rats administered $^3$H-secoisolariciresinol diglycoside ($^3$H-SDG) and a 10% flaxseed (FS) diet for 7 days. Bars with different letters (a-c) denote statistical significance among groups at p<0.05 by one-way ANOVA followed by Student-Newman-Keul’s post-hoc test for data that was normally distributed and had equal variances. For data that was not normally distributed (gastrointestinal tissues) or did not have equal variances (non-skeletal tissue), a Kruskal-Wallis one-way ANOVA followed by the Dunn’s post-hoc test was performed. Data are expressed as mean ± SEM. n=3 rats.
tissues (i.e. thymus, brain, uterus, vagina, bladder, spleen, lungs, liver, heart) (Figure 6-2B). Cecum radioactivity was 1.5 to 15.1 times higher than other gastrointestinal tissues (colon, stomach, small intestine). Radioactivity in the cecum and colon were significantly higher than in the small intestine (p<0.001), but they did not differ from each other (Figure 6-2C). Radioactivity of the stomach did not differ from other gastrointestinal tissues (Figure 6-2C). The radioactivity in serum was 7.8 ± 0.51 pmol/mL. In all tissues combined, the percentage of radioactivity recovered from the total administered dose was 5.6 ± 1.0%, mainly due to the radioactivity present in the whole liver, cecum and colon (1.1%-1.3% of administered dose), while the radioactivity recovered in all other whole tissues were below 1% of the total administered dose.

6.4 Discussion

This study has shown for the first time that SDG metabolites are accessible to skeletal tissue (i.e. LV, femurs and tibias) in the ovariectomized rat model of postmenopausal osteoporosis. While SDG metabolites are accessible to skeletal tissue and thus may have the potential to exert direct action on skeletal metabolism when combined with LD, it is unknown which metabolites (e.g. secoisolariciresinol, ED, EL, etc) may be responsible for these potential effects and, they are present at much lower levels than in most other tissues. This present study distinguishes itself from previous work because it is the first to determine whether SDG metabolites can be incorporated into skeletal tissue. Moreover, the current study was the first of its kind to administer $^3$H-SDG in combination with a 10% FS diet instead of unlabeled SDG, which is more reflective of the human diet.
FS and the metabolites of its major lignan (SDG) and n-3 PUFA (ALA), have been shown to favorably modulate skeletal metabolism (Babu et al., 2000; Griel et al., 2007; Feng et al., 2008). The up-regulation of proteins involved with bone formation (osteonectin, alkaline phosphatase and collagen I) was observed in MG-63 osteoblastic-like cells treated with SDG metabolites, ED and EL. In contrast, epidemiologic, in vivo and clinical studies have reported conflicting findings on the effectiveness of FS or its purified lignans on bone metabolism, particularly in the postmenopausal situation (Kardinaal et al., 1998; Kim et al., 2002; Lucas et al., 2002; Brooks et al., 2004; Dodin et al., 2005; Sacco et al., 2009b). Variability in experimental systems (e.g. animal versus humans; healthy adult rats versus ovariectomized rats representing a postmenopausal osteoporotic situation) may explain the conflicting findings regarding the effectiveness of FS or its components on bone metabolism. Even so, the only study investigating the effect of FS on bone metabolism in an ovariectomized rat model of postmenopausal osteoporosis observed that FS alone does not modulate BMD or bone strength (Study 1, Chapter 4) (Sacco et al., 2009b). Interestingly, the greatest benefit to vertebral BMD and strength was observed when FS was combined with LD (Study 1, Chapter 4) (Sacco et al., 2009b). These findings suggest that even though FS lignans and n-3 PUFA are accessible to skeletal tissue, their biological potency and level are not sufficient to induce a functional response on BMD or bone strength in ovariectomized when administered alone.

Whether the amount of SDG metabolites that reached the skeletal tissue is sufficient to modulate bone cell function when combined with LD requires further investigation. The level of radioactivity observed in the skeletal tissue analyzed in the current study represents 0.035% of the daily administered radioactivity. Based on Study 1 (Chapter 4) (Sacco et al., 2009b) that has shown that ovariectomized rats consume approximately 18 g of food per day, and based on the
amount of secoisolariciresinol aglycone present in our 10% FS diet (178.73 mg/kg food) used for this study (Study 1, Chapter 4) (Sacco et al., 2009b), we calculate that approximately 3.22 mg secoisolariciresinol was consumed daily by the rats in the present study, resulting in the accessibility of secoisolariciresinol metabolites to skeletal tissue at a level of 7.08 µg per gram of tissue throughout the 7-day study.

While the mechanisms whereby FS combined with LD preserves bone tissue in the ovariectomized rat model of postmenopausal osteoporosis are unknown, findings from the present study suggest that it may be possible for lignan metabolites to exert direct local action on bone metabolism. Like estrogen, the mammalian lignans (ED and EL) have the ability to bind to ERs, with preferential binding to ERα (Penttinen et al., 2007). ERα has been identified in osteoblasts, osteoclasts and their precursors; it regulates the transcription of a number of genes involved with bone turnover (OPG, M-CSF) (Komm et al., 2008). However, EL can also compete with estrogen for binding to the ER (Mueller et al., 2004) thus, may interfere with the effect of estrogen on skeletal metabolism. It is important to note in the present study that radioactive levels of SDG metabolites in skeletal tissue were relatively low, representing 4.0-8.2% of the concentration detected in the serum. This finding is similar to previous studies investigating the tissue distribution of selective ER modulators (SERMs) such as tamoxifen and raloxifene in which lower or minimal levels of these SERMs are reported in skeletal tissue compared to other selected tissues (Lien et al., 1991; Dodge et al., 1997).

In the present study, a small percentage of the administered radioactivity was found in tissues, which agrees with previous work that has shown that after 12 hours of 3H-SDG administration, most radioactivity is present in the cecal and colon contents (Rickard & Thompson, 1998). In contrast to others (Rickard & Thompson, 1998; Saarinen & Thompson,
2010), the current study observed that $^3$H-SDG metabolites in the serum was generally lower than that of most tissues excluding the skeletal and mammary tissues. These findings conflict with a previous study in which serum radioactivity was higher compared to all other tissues in 8-week-old intact female and male rats that were gavaged for one or seven days with $^3$H-SDG and unlabelled SDG (Saarinen & Thompson, 2010). Discrepancies between our findings with the previous study (Saarinen & Thompson, 2010) may be due to the level of $^3$H-SDG gavaged in the aforementioned study (3.7 kBq/g body weight), which is half of the dose that was used in the present study (7.4 kBq/g body weight). In addition, unlabelled SDG (5.3 μg/g bwt) in the previous study was gavaged to reflect the amount of SDG present in a 5% FS diet (Saarinen & Thompson, 2010), which contrasts to the present study whereby a 10% whole FS diet was administered. Thus, the higher dose of $^3$H-SDG and SDG from the 10% FS diet in the current study, along with other lignans (e.g. matairesinol, lari cereisol, pinoresinol) present in whole FS, could have resulted in greater intestinal absorption of $^3$H-SDG metabolites and radioactivity uptake in the tissues compared to serum. In fact, tissue levels of radioactivity in the current study are 4 to 8-fold higher than those reported in the aforementioned study (Saarinen & Thompson, 2010) with a similar distribution of radioactivity in commonly measured tissues. This suggests that tissues of both adult intact and ovariectomized female rats respond similarly to lignan intake. The previous study, in which both unlabeled and $^3$H-SDG were gavaged daily for one week in female adult rats, measured radioactivity only in non-skeletal tissues (Saarinen & Thompson, 2010).

Tritium labeling of SDG on the benzyl-methylene groups as opposed to the phenolic groups was performed because tritium is more stable at this site and, the benzyl-methylene groups are present only in known SDG metabolites (Rickard & Thompson, 1998). Previous
research using scintillation counting of HPLC fractions of urine from rats fed 3H-SDG, demonstrated that the radioactivity administered was only detected at the retention times for ED, EL, SECO and their other minor metabolites (Rickard & Thompson, 2000) which provides evidence that no hydrogen exchange occurred. Future study using scintillation counting of HPLC fractions from skeletal tissue is needed to determine whether this is also true in bone.

In conclusion, our study has shown for the first time that SDG metabolites are accessible to the LV, femurs and tibias of ovariectomized rats fed a 10% FS diet while being gavaged daily with $^3$H-SDG. However, whether SDG metabolites can exert direct action on bone cells and thus may play a role in the mechanism whereby FS combined with LD exerts a synergistic action on bone mass and strength in the ovariectomized rat model of postmenopausal osteoporosis requires further study.
CHAPTER 7
Study 4
7.0 Flaxseed Combined with Low-Dose Estrogen Therapy Reduces Bone Turnover in Ovariectomized Rats

7.1 Introduction

Estrogen attenuates postmenopausal bone loss by reducing resorption of bone. These effects on bone metabolism occur in part via regulation of the production of OPG and RANKL (Manolagas & Jilka, 1995; Bord et al., 2003; Syed & Khosla, 2005; Leibbrandt & Penninger, 2008). RANK, a transmembrane glycoprotein expressed in osteoclasts, is a receptor for RANKL that upon binding, stimulates the differentiation, survival and activity of bone-resorbing osteoclasts. OPG is the soluble decoy receptor for RANKL and as such blocks the interaction of RANKL and RANK and inhibits osteoclast formation, activity and survival (Leibbrandt & Penninger, 2008). Estrogen suppresses the production of RANKL indirectly by attenuating the production of pro-inflammatory cytokines such as TNFα and IL-6 (Suda et al., 1999). The action of estrogen on OPG production has been shown to be stimulatory in in vitro models and either stimulatory or inhibitory in ovariectomized rat models (Bord et al., 2003; Zhang et al., 2009; Bae & Kim, 2010; Luvizuto et al., 2010; Mok et al., 2010).

While the action of estrogen in preventing postmenopausal bone loss is primarily through the reduction of bone resorption, the mechanism(s) whereby FS combined with LD exerts the greatest protection to BMD, bone structure and biomechanical strength at the LV in ovariectomized rats, as observed in Study 1 (Chapter 4) (Sacco et al., 2009b), are currently unknown. Potential mechanisms include a reduction in osteoclast number and activity (bone resorption) via modulation of the OPG/RANKL/RANK axis as well as an increase in osteoblast number and activity.
In ovariectomized rats, a 10% FS oil diet has been shown to result in lower tartrate-resistant acid phosphatase-5\(\beta\) (TRAP-5\(\beta\)), a metalloenzyme secreted by osteoclasts, compared to controls suggesting that FS oil reduces osteoclast number (Boulbaroud et al., 2008). In addition, ALA has been shown to increase DNA content of MC3T3-E1 calvaria osteoblast cells suggesting that ALA stimulates proliferation of osteoblast lineage cells (Fujimori et al., 1989). The osteogenic potential of ED and EL have also been observed in MG-63 osteoblast-like cells whereby low levels of these lignans promote cell viability (Feng et al., 2008).

To understand the mechanisms by which FS acts in combination with LD exert the greatest protective effect on vertebra, the objective of this study was to determine how markers of bone resorption, bone formation and bone turnover are modulated by FS combined with LD. Markers of bone resorption and bone formation were measured in both the serum and at the local tissue level of the vertebral trabeculae. This strategy was used because serum analysis provides a fast and sensitive indication of the effects of various treatments on bone turnover while analysis of vertebrae provides information of treatment-induced effects that are specific to the skeletal site where the greatest protective effects of FS combined with LD on skeleton were previously observed (Study 1, Chapter 4) (Sacco et al., 2009b).

7.2 Methods

7.2.1 Animals and Diets

Animals and diets were as described in Chapter 4.

7.2.2 Experimental Design

Rats were acclimatized for one week on the BD after which they were randomized into the following groups (n=14/group): (1) negative (NEG) control fed the BD; (2) FS, fed BD
supplemented with 10% ground FS; (3) LD, fed BD and subcutaneously implanted with a 17β-estradiol pellet (13 µg, 90 day release; Innovative Research of America, Sarasota, FL, USA); and (4) FS+LD, fed the FS diet and implanted with a 17β-estradiol pellet. A sham-operated group fed the BD was included as a POS group. The pellets were titrated to mimic LD estrogen therapy (13 µg/day transdermal estrogen therapy) currently prescribed to postmenopausal women (North American Menopause Society, 2010b), and the pellets were placed subcutaneously in the interscapular region while rats were anesthetized with isoflurane (3%) dissolved in oxygen with a flow rate of 1 liter per minute. Buprenorphine (0.05 mg/kg body weight) was administered subcutaneously following surgery. After two weeks of treatment, rats were sacrificed by CO2 asphyxiation followed by cervical dislocation. The two-week study duration was chosen because treatment-induced effects on markers of bone metabolism occur rapidly in rat blood and skeletal tissue (Wronski et al., 1988; Sims et al., 1996; Zhang et al., 2007). Thus, we hypothesize that this time-point may serve as a window in which differences in markers of bone metabolism may explain the differences observed in BMD, bone structure and strength after chronic treatments (12 weeks) in Study 1 (Chapter 4) (Sacco et al., 2009b). Blood was obtained by cardiac puncture and centrifuged at 10000 G for 4 minutes to collect serum. Serum aliquots were stored at -80°C. LV1-3 were removed, cleaned of soft tissue, fixed in 10% buffered formalin for 24 hours, then stored at –20°C in 100% ethanol for histological analyses.

7.2.3 Serum Markers of Bone Turnover: TRAP-5β, CTX, Osteocalcin, OPG, RANKL, OPG/RANKL Ratio

To assess markers of bone resorption, serum levels of TRAP-5β, CTX, OPG and RANKL were measured. Enzyme-linked immunosorbent assay (ELISA) was used to measure serum TRAP-5β (RatTRAP™, Immunodiagnostic Systems Inc, Fountain Hills, AZ, USA) and CTX (RatLaps™,
Immunodiagnostic Systems Inc, Fountain Hills, AZ, USA) according to the manufacturer’s instructions. The lowest limits of detection for TRAP-5β and CTX were 0.1 U/L and 2.0 ng/mL, respectively. The intra-assay coefficients of variation for TRAP-5β and CTX were 0.30-9.12% and 1.74-9.21%, respectively. The inter-assay coefficients of variation for TRAP-5β and CTX were 0.50-5.96% and 0.13-4.17%, respectively.

For markers of bone formation, serum osteocalcin was analyzed using a Rat Bone Panel (Millipore, Billerica, MA, USA) with Luminex™ multiplex reagents according to the manufacturer’s instructions (Millipore, Billerica, MA, USA). Data were collected using Luminex 100 (Luminex Corporation, Austin, TX, USA). The lowest limit of detection was 1.6 pg/mL. The intra-assay coefficient of variation for osteocalcin was 0.37-0.87%.

To assess the OPG/RANKL/RANK axis, serum levels of OPG and RANKL were measured using a Rat OPG-Single Plex and a Rat RANKL-Single Plex from Millipore (Billerica, MA, USA) with Luminex™ multiplex reagents according to the manufacturer’s instructions (Millipore, Billerica, MA, USA). Data were collected using Luminex 100 (Luminex Corporation, Austin, TX, USA). The lowest limit of detection was 2.3 pg/mL for OPG and 1.0 pg/mL for RANKL. The intra-assay coefficients of variation for OPG and RANKL were 1.01-2.60% and 2.68-5.04%, respectively, and the inter-assay coefficients of variation for OPG and RANKL were 16.07% and 9.67%, respectively. The OPG/RANKL ratio was determined based on serum levels of OPG and RANKL for each rat.

7.2.4 Morphological and Immunohistochemical Evaluation of Bone Turnover in Lumbar Vertebrae: Hematoxylin and Eosin, TRAP-5β, Osterix, Osteocalcin, OPG, RANKL

Morphological evaluation of LV5 was performed on hematoxylin and eosin stained sections. Markers of bone resorption (TRAP-5β, OPG, RANKL) and bone formation (osterix, osteocalcin)
in LV5 were evaluated by enzymatic or immunohistochemical staining. Decalcification in 10% EDTA solution, paraffin-embedding, sectioning (5 µm per section), hematoxylin and eosin and TRAP-5β staining of formalin-fixed LV were performed at the Centre for Bone and Periodontal Research, McGill University, Montreal, QC, Canada. For OPG, RANKL, osterix and osteocalcin staining, 5µm sections were deparaffinized, rehydrated and endogenous peroxidase was blocked with aqueous 3% H₂O₂. The antigen was retrieved by heating in Tris-EDTA buffer at pH 6.0 for 20 minutes in a microwave oven. The primary antibodies were diluted in Diluent Buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA), made of 0.05 M Tris-HCl buffer containing 0.1% Tween, 0.015 M sodium azide and stabilizing proteins to reduce background. Samples were incubated overnight at 4°C with goat-polyclonal anti-OPG (sc-8468, Santa Cruz Biotechnology, Santa Cruz, CA USA), goat-polyclonal anti-RANKL (sc-7627, Santa Cruz Biotechnology, Santa Cruz, CA USA), rabbit-polyclonal anti-Sp7/osterix (ab22552, Abcam, Cambridge, MA, USA), and rabbit-polyclonal anti-osteocalcin (sc-30044, Santa Cruz Biotechnology, Santa Cruz, CA US). After washing in phosphate-buffered saline (3x 5 min), samples were incubated with donkey anti-goat (sc-2042, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or goat anti-rabbit (sc-2040, Santa Cruz Biotechnology, Santa Cruz, CA, USA) secondary antibodies. Streptavidin-HRP and AEC substrate chromagen were used to demonstrate the antigens. Control sections were stained with antibody diluent instead of the primary antibodies, and processed in the same manner as all other samples. All agents were obtained from Dako (Mississauga, ON, Canada). Slides were evaluated by light microscopy (BX51 microscope, Olympus, Center Valley, PA, USA) under 100x magnification for morphological evaluation and 400x magnification for immunohistochemical evaluation in a double-blinded fashion. Images were obtained with a SPOT RT camera (Diagnostic Instruments,
Sterling Heights, MI, USA) and by using Image-Pro Plus software (Media Cybernetics Inc, Bethesda, MD, USA).

For determination of osteoclast number per microscopic field, osteoclasts with positive stains for TRAP-5β were counted in six randomly selected fields of the trabecular bone of LV5 at 400x. For determination of osteoblast number per field, mononuclear osterix-positive cells adjacent to the trabecular surface were counted in six randomly selected fields at 400x. The average number of osteoclasts and osteoblasts per field in each group was then determined at 400x. Osteoblasts expressing OPG, RANKL and osteocalcin were evaluated by determining the sum of an intensity score, ranging from 0 (negative) to 3 (strong staining), and a proportion score, ranging from 0 (0% positive) to 5 (100% positive), with a maximum score of 8 (range 0-8) (Allred et al., 1998).

### 7.2.5 Statistical Analyses

Statistical analyses were performed to detect differences among groups for serum levels of TRAP-5β, CTX, osteocalcin, and expression of TRAP5β, osterix, osteocalcin, OPG, RANKL and OPG/RANKL ratio in LV5. Differences among groups were determined by one-way ANOVA followed by the Student-Newman Keul’s post-hoc test (SigmaStat; Systat Software Inc, Version 3.5, Chicago, IL, USA). A Kruskal-Wallis one-way ANOVA followed by the Dunn’s post-hoc test was used for data that were not normally distributed (serum RANKL, serum OPG/RANKL ratio, LV5 OPG). Results are expressed as mean ± SEM. The significance level was set at \( p < 0.05 \).
7.3 Results

7.3.1 Serum Markers of Bone Turnover

7.3.1.1 TRAP-5β

TRAP-5β was significantly lower in the NEG compared to the POS group (p<0.001) (Figure 7-1A) as expected, since the total number of osteoclasts in bone tissue decreases after ovariectomy due to substantial loss in the amount of bone tissue caused by the decline in estrogen (Alatalo et al., 2003; Chu et al., 2003). There were no significant differences in serum TRAP-5β observed between the NEG and FS groups, while the LD and FS+LD resulted in TRAP-5β levels that were significantly lower than both the NEG and FS groups (p<0.05). No differences were observed between the LD and FS+LD groups (Figure 7-1A).

7.3.1.2 CTX

CTX was significantly higher in NEG compared to the POS group (p<0.001) (Figure 7-1B) as expected, since the activity of osteoclasts and the resorption of bone increases after ovariectomy. There were no significant differences in serum CTX observed between the NEG and FS groups, while the LD and FS+LD resulted in CTX levels that were significantly lower than both the NEG and FS groups and similar to the POS group (p<0.001). No differences were observed between the LD and FS+LD groups (Figure 7-1B).

7.3.1.3 Osteocalcin

Osteocalcin was significantly lower in POS compared to the NEG group (p<0.01) (Figure 7-1C), confirming that bone formation is lower in ovary-intact rats compared to ovariectomized rats. There were no significant differences in serum osteocalcin between the NEG and FS groups while LD and FS+LD resulted in osteocalcin levels that were significantly lower than both the
Figure 7-1: Effect of 10% dietary flaxseed (FS), low-dose estrogen therapy (LD) and their combination (FS+LD) on serum levels of tartrate resistant acid phosphatase-5β (TRAP-5β) (A), C-terminal telopeptides of Type I collagen (CTX) (B), osteocalcin (C), osteoprotegerin (OPG) (D), receptor activator of nuclear factor κβ (RANKL) (E), and OPG/RANKL ratio (F) in ovariectomized rats.

POS= positive control, NEG= negative control. LD= 13 µg, 90-day release. Bars with different letters (a-c) indicate a significant difference among groups at p<0.05 by one-way ANOVA followed by Student-Newman Keul’s post-hoc test for data that was normally distributed (TRAP-5β, CTX, osteocalcin) or by Kruskal-Wallis one-way ANOVA followed by the Dunn’s post-hoc test for data that was not normally distributed (RANKL, OPG/RANKL ratio). Data are expressed as mean ± SEM. n=12-14 rats/group (TRAP, CTX, OPG, RANKL, OPG/RANKL ratio), n=6-8 rats/group (osteocalcin).
NEG and FS groups (p<0.01) but similar to the POS group. There were no differences in serum osteocalcin levels between the LD and FS+LD groups (Figure 7-1C).

7.3.1.4 OPG

OPG was significantly lower in NEG compared to the POS group (p<0.05) (Figure 7-1D). FS resulted in significantly higher serum OPG levels compared to NEG (p<0.05) while LD and FS+LD resulted in the highest OPG levels which were similar to POS (p>0.05) and significantly higher than both the NEG and FS groups (p<0.05). No differences in serum OPG were observed between the LD and FS+LD groups (Figure 7-1D).

7.3.1.5 RANKL

RANKL was significantly higher in the NEG and FS groups compared to the POS group (p<0.05) and did not differ from each other (Figure 7-1E). LD resulted in the lowest serum RANKL levels compared to all other groups (p<0.05) while FS+LD induced serum RANKL levels that were intermediate to the NEG, FS and LD groups (p<0.05) and did not differ from POS (p>0.05) (Figure 7-1E).

7.3.1.6 OPG/RANKL Ratio

OPG/RANKL ratio was significantly lower in NEG compared to the POS group (p<0.05) (Figure 7-1F). There were no significant differences between FS and NEG, while LD and FS+LD resulted in serum OPG/RANKL ratios that were significantly higher than the NEG and FS groups (p<0.05) but similar to POS group. No significant differences in serum OPG/RANKL ratios were observed between the POS, LD and FS+LD groups (Figure 7-1F).
7.3.2 Morphological and Immunohistochemical Evaluation of Bone Turnover at the Lumbar Vertebrae

7.3.2.1 Morphology of LV5

Phenotypical differences in trabecular properties of LV5 were visualized semi-quantitatively (Figure 7-2A) and qualitatively (Figure 7-2B) using hematoxylin and eosin staining. The POS group resulted in a significantly higher (p<0.05) bone volume fraction compared to the NEG and FS groups, while LD did not differ from either the POS and NEG groups. FS+LD resulted in a bone area fraction that was significantly higher (p<0.05) than the NEG group and did not differ from POS. The POS group demonstrated normal structure of trabecular bone, while the NEG group demonstrated evidence of ovariectomy-induced bone loss as well as lower trabecular bone volume and fewer trabeculae were evident compared to POS. Moreover, the separation between trabeculae appeared to be greater in the NEG group compared to the POS group. FS and LD treatments resulted in LV5 structures that were intermediate to the POS and NEG groups in terms trabecular number and separation. FS+LD treatment resulted in trabecular phenotypes that resembled most closely to that of the POS compared to all other treatments (Figure 7-2).

7.3.2.2 TRAP-5β

TRAP-5β at the trabecular surface was significantly higher in the POS group compared to the NEG group (p<0.01) (Figure 7-3A, Figure 7-3B). FS and LD resulted in TRAP-5β similar to NEG while a trend was observed for FS+LD to result in higher TRAP-5β than NEG (p=0.08). Moreover, LD and FS+LD resulted in TRAP-5β that were similar to the POS group (Figure 7-3A, Figure 7-3B).
Figure 7-2: Effect of 10% dietary flaxseed (FS), low-dose estrogen therapy (LD) and their combination (FS+LD) on bone volume fraction (A) and morphology of the lumbar vertebra 5 (LV5) (B) in ovariectomized rats.

POS = positive control, NEG = negative control. LD = 13 µg, 90-day release. The appearance of lower trabecular number and greater trabecular separation in NEG group compared to the POS group (depicted by arrows) is characteristic of trabecular bone in ovariectomized rats. The trabecular characteristics in the FS and LD groups appear intermediary to the POS and NEG groups however, the greatest resemblance to the POS group occur in the FS+LD group. Bars with different letters (a-b) indicate a significant difference among groups at p<0.05 by one-way ANOVA followed by Student-Newman Keul’s post-hoc test. Data are expressed as mean ± SEM. n= 8 rats/group. 100x magnification.
Figure 7-3: Effect of 10% dietary flaxseed (FS), low-dose estrogen therapy (LD) and their combination (FS+LD) on expression of tartrate resistant acid phosphatase 5β (TRAP-5β) (A) and immunolocalization of TRAP-5β in lumbar vertebra 5 (LV5) (B) in ovariectomized rats.

POS= positive control, NEG= negative control. LD implant= 13 µg, 90-day release. Arrows depict areas of positive staining for TRAP-5β. Bars with different letters (a-b) indicate a significant difference among groups at p<0.05 by one-way ANOVA followed by Student-Newman Keul’s post-hoc test. Data are mean ± SEM. n= 8 rats/group. 400x magnification.
7.3.2.3 Osterix

Osteoblast expression of osterix was significantly higher in the POS group compared to the NEG group (p<0.05) (Figure 7-4A, Figure 7-4B). FS, LD and FS+LD resulted in significantly higher expression of osterix compared to the NEG (p<0.05), and did not differ from POS.

7.3.2.4 Osteocalcin

Osteoblast expression of osteocalcin was significantly higher in the NEG group compared to the POS and FS+LD groups (p<0.05) (Figure 7-5A, Figure 7-5B). No other differences in osteocalcin expression were observed.

7.3.2.5 OPG

Osteoblast expression of OPG in LV5 was significantly lower in NEG compared to POS and FS+LD groups (p<0.001) (Figure 7-6A, Figure 7-6B). No other differences in OPG expression were observed among groups.

7.3.2.6 RANKL

RANKL expression in osteoblasts were significantly higher in the NEG group compared to the POS group (p<0.05) (Figure 7-7A, Figure 7-7B), while no other differences in RANKL expression were observed among groups.

7.4 Discussion

The results from this study (Table 7-1) demonstrate that two weeks administration of FS combined with LD attenuates ovariectomy-induced bone turnover as observed by lower serum TRAP-5β, CTX and osteocalcin compared to the NEG group. In addition, expression of TRAP-5β, osterix and osteocalcin did not differ between the FS+LD and POS groups at the vertebra providing evidence that the combination of FS and LD protects against ovariectomy-induced
Figure 7-4: Effect of 10% dietary flaxseed (FS), low-dose estrogen therapy (LD) and their combination (FS+LD) on protein expression of osterix (A) and immunolocalization of osterix in lumbar vertebra 5 (LV5) (B) in ovariectomized rats. POS= positive control, NEG= negative control. LD implant= 13 µg, 90-day release. Arrows depict areas of positive staining for osterix. Bars with different letters (a-b) indicate a significant difference among groups at p<0.05 by one-way ANOVA followed by Student-Newman Keul’s post-hoc test. Data are mean ± SEM. n= 7-8 rats/group. 400x magnification.
Figure 7-5: Effect of 10% dietary flaxseed (FS), low-dose estrogen therapy (LD) and their combination (FS+LD) on protein expression of osteocalcin (A) and immunolocalization of osteocalcin in lumbar vertebra 5 (LV5) (B) in ovariectomized rats.

POS = positive control, NEG = negative control. LD implant = 13 µg, 90-day release. Arrows depict areas of positive staining for osteocalcin. Bars with different letters (a-b) indicate a significant difference among groups at p<0.05 by one-way ANOVA followed by Student-Newman Keul’s post-hoc test. Data are mean ± SEM. n= 7-8 rats/group. 400x magnification.
Figure 7-6: Effect of 10% dietary flaxseed (FS), low-dose estrogen therapy (LD) and their combination (FS+LD) on serum levels of osteoprotegerin (OPG) (A) and immunoreactivity for OPG in lumbar vertebra 5 (LV5) (B) in ovariectomized rats.

POS= positive control, NEG= negative control. LD implant= 13 µg, 90-day release. Arrows depict areas of positive staining for OPG. Bars with different letters (a-b) indicate a significant difference among groups at p<0.05 by Kruskal-Wallis one-way ANOVA followed by the Dunn’s post-hoc test. Data are mean ± SEM. n= 7-8 rats/group. 400x magnification.
Figure 7-7: Effect of 10% dietary flaxseed (FS), low-dose estrogen therapy (LD) and their combination (FS+LD) on serum levels of receptor activator of nuclear factor κβ (RANKL) (A) and immunoreactivity for RANKL in lumbar vertebra 5 (LV5) (B) in ovariectomized rats.

POS= positive control, NEG= negative control. LD implant= 13 µg, 90-day release. Arrows depict areas of positive staining for RANKL. Bars with different letters (a-b) indicate a significant difference among groups at p<0.05 one-way ANOVA followed by Student-Newman Keul’s post-hoc test. Data are mean ± SEM. n= 8 rats/group. 400x magnification.
Table 7-1: Summary on the effects of flaxseed (FS), low-dose estrogen therapy (LD) and their combination on markers of bone turnover in the serum and lumbar vertebrae 5 (LV5) compared to negative control (NEG)

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bone loss and deterioration of bone tissue through modulation of bone cell numbers and bone cell activities. However, the observation that FS+LD did not differ from LD in multiple measures of bone turnover suggests that further investigation is required to clarify how FS+LD modulates bone turnover to result in greater protection to BMD, bone structure and strength at the LV.

After two weeks of treatment with FS+LD, serum TRAP-5β and CTX were lower compared to the NEG and these results correspond to the protection to LV trabecular structure in the current study as observed by a greater bone volume fraction and, to the preservation of LV BMD, bone structure and strength observed in Study 1 (Chapter 4) (Sacco et al., 2009b). No research on the effects of FS combined with estrogen therapy on biochemical markers of bone metabolism has been previously performed, and little is known regarding the effects of FS alone. In one study using ovariectomized rats fed a 10% FS oil diet for four weeks, lower serum TRAP-5β was observed compared to the NEG group (Boulbaroud et al., 2008). In contrast, short-term (3-4 months) studies in healthy postmenopausal women consuming 25-40 g FS per day have shown that serum alkaline phosphatase, TRAP and urinary deoxypyridinoline levels do not differ from the wheat-based placebo group (Lucas et al., 2002; Brooks et al., 2004). The results from Lucas et al (2002) and Brooks et al (2004) agrees with the findings in the present study which observed no differences in serum levels of TRAP-5β, CTX and osteocalcin between FS and NEG groups. Discrepancies between the current study and that of Boulbaroud et al (2008) may be due to the oil content of the diets. The 10% FS level in the current study provided 2.36g ALA per kg of diet, whereas the 10% FS oil diet provided approximately 24g ALA per kg of diet. Despite the fact that the FS group had higher serum levels of OPG with no differences in the level of
RANKL or the ratio of OPG to RANKL compared to the NEG group, it may be that a diet with higher levels of ALA is required to modulate bone resorption in ovariectomized rats.

The observation that LD reduces serum biochemical markers of bone turnover (TRAP-5β, CTX, osteocalcin) in the current study agrees with previous research of estrogen treatment in ovariectomized rats and in postmenopausal women (Peris et al., 1999; Gopala Krishnan et al., 2003; Delmas et al., 2004; Mukherjee et al., 2006; Huang et al., 2007; Poulsen et al., 2008). In contrast, similar levels of serum biochemical markers of bone turnover were observed between the LD and FS+LD, which contradicts the observations that FS+LD induces greater protection than LD against the deterioration of bone tissue in ovariectomized rats (Study 1, Chapter 4) (Sacco et al., 2009b). The single observed difference in serum markers of bone turnover was that FS+LD induced greater serum levels of RANKL compared to LD suggesting that FS+LD may stimulate osteoclast formation and activity. However, the ratio of OPG/RANKL is a major determining factor of osteoclast formation and activity and, it did not differ between groups. It is possible that other markers of bone turnover (osteopontin, type I collagen, NTX, deoxypyridinoline) may distinguish the difference between the actions of FS+LD and LD on the vertebra in ovariectomized rats. It is also possible that the study duration played an important factor in determining differences between groups. Markers of bone turnover were assessed after two weeks of treatment with FS, LD and FS+LD. It is possible that differences between treatments may occur earlier (e.g. one week post treatment initiation) or later than the chosen two-week period. Since markers of bone turnover are routinely used to predict subsequent bone loss and, differences in microarchitecture were already observed after two weeks of treatment in the current study, it may be that differences in markers of bone turnover were more pronounced closer to the initiation of the treatments.
While serum biochemical markers of bone turnover are useful for the prediction of bone loss during the early stages post-ovariectomy and for monitoring the effectiveness of antiresorptive treatments, they reflect bone turnover of the whole skeleton rather than specific skeletal sites. Thus, analysis of TRAP-5β, osteocalcin, OPG and RANKL expression was also performed in LV using histological staining. Protein expression of osterix was also determined through histological staining of LV since it is expressed in osteoblast cells (Nakashima et al., 2002) and thereby can provide an indication of the differences in osteoblast numbers between groups. Semi-quantitative analysis at the LV demonstrated that while FS+LD induces higher osterix expression compared to the NEG and FS groups, it did not differ from the LD group suggesting that the enhanced protection to LV bone mass, structure and strength observed when FS is combined with LD compared to LD alone (Study 1, Chapter 4) (Sacco et al., 2009b) may not be due to the modulation of osteoblast numbers. Instead, FS+LD resulted in an osteocalcin score that was significantly lower than the NEG group while LD did not. Furthermore, FS+LD resulted in an OPG score that was significantly higher than the NEG group while LD did not. These results indicate that there may be a small advantage in reducing bone turnover when FS is combined with LD.

FS is rich in SDG and ALA, compounds that are implicated in modulating bone turnover. The mammalian lignans produced through the consumption of SDG can exert estrogenic and antiestrogenic effects in various estrogen-sensitive tissues such as the mammary gland and uterus. However, EL has been shown to exert no estrogenic effect on skeletal tissue of ovariectomized transgenic estrogen-sensitive reporter mice (Penttinen et al., 2007). It is possible that other metabolites of SDG such as secoisolariciresinol and ED exert estrogenic action on skeletal tissue however, future research is required to determine whether they can exert
estrogenic action on skeletal tissue, or whether they along with EL can modulate signaling pathways that are independent of the ER. In addition, the direct effect of ALA on skeletal tissue is undetermined. However, diets deficient of n-3 PUFA have been shown to cause severe osteoporosis. In ovariectomized Fat-1 mice, which can endogenously synthesize n-3 PUFA, lower serum levels of RANKL and TRAP-5β were observed compared to wild-type mice (Rahman et al., 2009). In addition, activation of NF-κB and lower activity of COX-II was observed in Fat-1 mice compared to the wild-type controls suggesting that n-3 PUFA may attenuate osteoclastogenesis (Rahman et al., 2009). It is suggested that the major actions of the n-3 PUFA on skeletal tissue are through the action of the longer-chain metabolites of ALA such as EPA and DHA, as multiple studies using ovariectomized rats have observed a protective effect of these fatty acids on skeletal tissue (Watkins et al., 2006; Poulsen et al., 2007; Poulsen et al., 2008).

In the present study, discrepancies in markers of bone metabolism were observed between the serum and LV. For example, FS resulted in higher serum OPG levels compared to NEG while in the LV, no differences were observed. LD and FS+LD also induced greater effects in the serum than in the LV. However, FS+LD maintained its stimulatory effect on OPG and inhibitory effect on osteocalcin compared to NEG demonstrating some consistencies in results. A number of factors may contribute to these findings, including differences in treatment-induced responses among various skeletal sites and differences in sensitivities of the serum and histological analyses.

In conclusion, FS combined with LD attenuates ovariectomy-induced bone turnover in both the serum and in the LV, which may be in part due to its action on the OPG/RANK/RANKL axis. In addition, there may be a small advantage in reducing bone
turnover when FS is combined with LD compared to LD alone as observed by lower osteocalcin and higher OPG with the combined treatment. However, the observation that markers of bone turnover did not differ between FS+LD and LD suggests that the mechanisms whereby FS+LD result in a greater protection to bone mass, bone structure and bone strength at the LV requires further investigation.
Uterus tissue was collected from: Sacco SM, Jiang JM, Reza-Lopez S, Ma DW, Thompson LU, Ward WE. Flaxseed combined with low-dose estrogen therapy preserves bone tissue in ovariectomized rats. Menopause. 2009;16(3):545-554. Uteri analyses were performed subsequent to publication of Sacco et al (2009b).
8.0 Effect of Flaxseed, Alone or Combined With Low-Dose Estrogen Therapy, on Uterus Health in Ovariectomized Rats

8.1 Introduction

Estrogen replacement therapy is effective for the treatment of postmenopausal-related conditions such as bone loss (Rossouw et al., 2002; Anderson et al., 2004). However, findings from the Women’s Health Initiative (WHI) trial reported that its use is associated with a number of adverse side effects including coronary events, pulmonary embolism and breast cancer (Rossouw et al., 2002; Anderson et al., 2004). It is believed that some of the adverse side effects observed in the WHI trial are related in part to the addition of progestin since a greater number of adverse effects were observed in the combination arm (CEE+MPA) compared to the estrogen monotherapy arm (CEE). However, the inclusion of progestin with estrogen therapy is required for women with an intact uterus using standard dose (0.625 mg/d) CEE for the prevention of uterine hyperplasia and carcinoma (Grady et al., 1995).

Currently, lower doses of estrogen therapy without the addition of progestin are being investigated for the treatment of postmenopausal-related conditions including prevention of postmenopausal bone loss. Findings using half and one-quarter the standard dose of estrogen through the transdermal delivery system show that lower doses of estrogen monotherapy prevent bone loss and do not stimulate uterine hyperplasia (Ettinger et al., 2004; Richman et al., 2006; Ettinger, 2007; Samsioe et al., 2007). Thus, side effects associated with the addition of progestin can be avoided with lower doses of estrogen monotherapy, and their clinical use can also be made relevant to postmenopausal women with an intact uterus. Nevertheless, many postmenopausal women are seeking for more natural therapies for the support of menopausal health either as alternatives or complements to pharmacological therapy because they are viewed
as safer than currently available pharmacological options (Mahady et al., 2003; Grady, 2006). FS, rich in the lignan SDG and the n-3 PUFA ALA, is an example of a commonly consumed natural health product that is consumed for the postmenopausal support and the prevention of chronic diseases.

As discussed in Section 2.4.1.1, SDG is a phytoestrogen that is present in very high amounts in FS and in the Western diet (Mazur, 1998; Mazur et al., 1998; Chen et al., 2006) and once consumed, it is metabolized by intestinal microflora into the more biologically active mammalian lignans, ED and EL. Because the mammalian lignans share structural similarity to the steroid hormone 17ß-estradiol, they can have estrogenic properties in hormone-sensitive tissues such as the uterus (Penttinen et al., 2007). Thus, the mammalian lignans have the potential to modulate the risk of uterine hyperplasia and carcinoma. However, a study in postmenopausal women that provided 25g of ground FS a day for 12 weeks observed that FS did not induce endometrial hyperplasia (Simbalista et al., 2010). Similar findings have been reported using rat and mouse models (Chen et al., 2003; Chen et al., 2006; Power et al., 2006; Chen et al., 2007; Penttinen-Damdimopoulou et al., 2009) whereby feeding ground FS or purified SDG did not stimulate uterine wet weight, a crude measurement of endometrial hyperplasia or morphology. Because Study 1 (Chapter 4) (Sacco et al., 2009b) observed that FS+LD exerts the greatest protection to vertebral BMD, structure and strength compared to either treatment alone, it is of research interest to determine their combined effects on markers of uterine health. Since dietary FS has been previously shown to not stimulate uterine tissue (Chen et al., 2003; Chen et al., 2006; Power et al., 2006; Chen et al., 2007; Penttinen-Damdimopoulou et al., 2009; Simbalista et al., 2010), it is hypothesized that FS, alone or combined with estrogen therapy, will not adversely affect uterine morphology or cell proliferation compared to LD alone.
Thus, the objective of this study was to evaluate the effect of FS, alone or combined with LD, on general uterine morphology and cell proliferation using the ovariectomized rat model of postmenopausal osteoporosis.

8.2 Methods

8.2.1 Animals and Diets

Animals and diets were as described in Chapter 4 (Sacco et al., 2009b).

8.2.2 Experimental Design

The experimental design was as described in Chapter 4 (Sacco et al., 2009b). Briefly, rats were acclimatized for one week on the basal diet (BD) after which they were randomized into the following groups (n=12/group): (1) negative (NEG) control fed the BD; (2) FS, fed BD supplemented with 10% ground FS; (3) LD, fed BD and subcutaneously implanted with a 17β-estradiol pellet (13 μg, 90 day release; Innovative Research of America, Sarasota, FL, USA); and (4) FS+LD, fed the FS diet and implanted with a 17β-estradiol pellet. A sham-operated group fed the BD was included as a POS group. The pellets were titrated to mimic LD transdermal estrogen therapy (25 µg/day 17β-estradiol) currently prescribed to postmenopausal women (North American Menopause Society, 2010b), and the pellets were placed subcutaneously in the interscapular region while rats were anesthetized with isoflurane (3%) dissolved in oxygen with a flow rate of 1 liter per minute. The analgesic buprenorphine (0.05 mg/kg body weight) was administered subcutaneously following surgery. Rats were sacrificed after twelve weeks that is approximately equivalent in duration to a one-year clinical trial in postmenopausal women (Baron et al., 1984; Kimmel et al., 1990; U.S. Food and Drug Administration, 1994) to evaluate chronic effects of FS, LD and FS+LD on uterine health. Sacrifice was performed by CO₂
asphyxiation followed by cervical dislocation and wet uterus weight was determined. Uteri were then fixed in 10% neutral buffered formalin for future histological analyses.

### 8.2.3 Morphological and Immunohistochemical Evaluation of Uteri

Formalin-fixed uteri were dehydrated in increasing grades of ethanol and then embedded in paraffin for sectioning. 5µm slices were mounted on microscopic glass slides coated with poly-L-lysine then deparaffinized and rehydrated. Hematoxylin and eosin staining was performed for qualitative evaluation of general uterine morphology (epithelial mass, structure of luminal epithelial layer). For evaluation of cell proliferation, expression of proliferating cell nuclear antigen (PCNA) was determined at the luminal epithelial layer of the uterus. Endogenous peroxidase was blocked with aqueous 3% H₂O₂. Microwave heating of samples in 0.01 M citrate buffer at pH 6.0 for 20 minutes was used to retrieve the antigen. The mouse anti-rat PCNA (sc-56, Santa Cruz Biotechnology Inc. Santa Cruz, CA, USA), was diluted in Diluent Buffer to block non-specific antigens, then incubated overnight at 4ºC. Samples were then incubated in biotinylated anti-mouse IgG (K0609, Dako, Mississauga, ON, Canada). Streptavidin-HRP and AEC substrate chromagen were used to demonstrate the antigens. All immunostaining agents were purchased from Dako (Mississauga, ON, Canada), and the slides were read blindly under a light microscope at 400x magnification. 20-500 luminal epithelial cells from 3-6 fields per uterus were counted, and the PCNA labeling index was calculated as percentage of positive cells over total number of cells counted.

### 8.2.4 Statistical Analyses

Differences between groups were determined by one-way ANOVA followed by the Student-Newman Keul’s post-hoc test (SigmaStat; Systat Software Inc, Version 3.5, Chicago, IL, USA). Results are expressed as mean ± SEM. The significance level was set at \( p < 0.05 \).
8.3 Results

8.3.1 Uterus Weight

The POS group resulted in significantly higher (p<0.001) relative uterus weight compared to all other groups (Figure 8-1). No other differences in uterus weight were observed between groups.

8.3.2 General Morphological Characteristics of Uteri

Qualitative histological analysis of the uteri demonstrates normal histology of intact rats in the POS group (Figure 8-2). The POS group exhibited higher epithelial mass compared to all other groups. Luminal epithelia in the NEG group were flattened or cuboidal cells organized in a single layer. FS, LD and FS+LD treatments resulted in a single layer of elongated luminal epithelia that were columnar in shape (Figure 8-2).

8.3.3 PCNA Labeling Index of Luminal Epithelial Cells

The POS group had significantly higher (p<0.05) cell proliferation in the luminal epithelium compared to the NEG control group. Similarly, the POS group resulted in significantly higher (p<0.05) cell proliferation compared to the FS, LD and FS+LD groups (Figure 8-3A, Figure 8-3B). No other differences in cell proliferation were observed between groups.

8.4 Discussion

This study is the first to demonstrate the effects of FS, alone and combined with LD, on uterus of the ovariectomized rat model of postmenopausal osteoporosis. The results of the present study demonstrate that FS, LD and FS+LD do not exert stimulatory effects on uterus weight and cell proliferation of the luminal epithelium compared to the NEG group. Moreover, uterus weight and cell proliferation were significantly lower in all the ovariectomized groups compared to the
Figure 8-1: Effect of 10% dietary flaxseed (FS), alone and combined with low-dose estrogen therapy (LD) on uterus wet weight in ovariectomized rats.

POS= Positive Control, NEG= Negative Control. LD implant= 13 µg, 90-day release. Bars with different letters (a-b) indicate a significant difference among groups at p<0.05 by one-way ANOVA followed by Student-Newman Keul’s post-hoc test. Data are mean ± SEM. n= 11-12 rats/group.
Figure 8-2: Effect of 10% dietary flaxseed (FS), alone and combined with low-dose estrogen therapy (LD) on luminal epithelial phenotype in ovariectomized rats.
POS= positive control, NEG= negative control. LD implant= 13 µg, 90-day release. The appearance of a single layer of flattened or cuboidal epithelial cells in NEG group and a lower epithelial mass compared to the POS group (depicted by arrows) is characteristic of the luminal epithelium in ovariectomized rats. The epithelial characteristics of FS, LD and FS+LD consist of a single layer of elongated cells that are columnar in shape. n= 6-8 rats/group. 400x magnification.
Figure 8-3: Effect of 10% dietary flaxseed (FS), alone and combined with low-dose estrogen therapy (LD) on proliferating cell nuclear antigen (PCNA) labeling index (A) and immunoreactivity for PCNA (B) in luminal epithelial uterine cells in ovariectomized rats. POS= Positive Control, NEG= Negative Control. LD implant= 13 µg, 90-day release. Examples of immunoreactivity in luminal epithelial cells are marked with arrows. Different letters (a-b) indicate a significant difference among groups at p<0.05 by one-way ANOVA followed by Student-Newman Keul’s post-hoc test. Data are mean ± SEM. n= 5-8 rats/group. 400x magnification.
POS group, demonstrating ovariectomy-induced atrophy of the uterus tissue. While FS, LD and FS+LD similarly demonstrated an estrogenic effect on the morphology of the luminal epithelia (greater epithelial mass, elongated columnar cells) compared to the NEG control, these treatment-induced effects were lower in magnitude compared to the POS group. Since estrogen exerts a dose-dependant effect on the risk of endometrial hyperplasia and carcinoma on women with an intact uterus (Grady et al., 1995; Lindsay et al., 2002), findings from this study suggest that SDG-rich FS does not modulate the effect of LD on uterine tissue in ovariectomized rats.

The observation that feeding whole ground FS alone, and when combined with LD, do not induce higher uterine weight or cell proliferation compared to the ovariectomized control group (NEG) in the current study suggests that FS does not induce uterotrophic effects and, that it does not modulate the potential effect of LD in ovariectomized rats. While no studies have investigated the combined effects of FS and HT on markers of uterine health, the observations of FS on the uterus in the current study agree with previous research in humans and rodent models (Bergman Jungestrom et al., 2007; Power et al., 2007; Chen et al., 2009; Penttinen-Damdimopoulou et al., 2009; Simbalista et al., 2010; Chen et al., 2011). Daily consumption of 25g ground FS in bread for 12 weeks did not induce endometrial hyperplasia in young postmenopausal women with climacteric symptoms (Simbalista et al., 2010). Studies using ovariectomized athymic nude mice with estrogen responsive breast cancer also observed no uterotrophic effect from feeding a diet consisting of 10% FS or, FS hull, SDG, ED or EL at a level equivalent to a 10% FS diet (Chen et al., 2004; Power et al., 2006; Power et al., 2007; Chen et al., 2009). In ovariectomized transgenic estrogen-sensitive reporter mice exposed to a 14% FS diet for 40 hours or 14 days, no differences in uterine weights or morphological indices were observed compared to the control (Penttinen-Damdimopoulou et al., 2009). In contrast,
research using this same mouse model has demonstrated that exposure to a one-time injection of EL at a level equivalent to a 10% FS diet exerts stromal edema in 29% of the mice 12 hours post-injection however, at 24 hours post-injection, this effect disappeared (Penttinen et al., 2007). While an increase in cyclin D1 expression was also observed in the uterine epithelium and stroma of the reporter mice, higher Ki-67 expression (a marker of cell proliferation) was observed in the glandular epithelium but not in the luminal epithelium or stroma 24 hours post-injection (Penttinen et al., 2007). Thus, long-term effects of EL were small (Penttinen et al., 2007) and may not be reflective of a diet consisting of whole FS.

Our finding that LD did not induce uterotrophic effects is consistent with previous research (Genant et al., 1997; Samsioe et al., 2007). However, others have demonstrated that LD is associated with an increase in the incidence of endometrial cancer (Cushing et al., 1998; Furness et al., 2009). Discrepancies in findings may be related to differences in study designs, method of delivery and duration of therapy. In the studies by Genant et al., 1997 and Samsioe et al., postmenopausal women received 0.3 mg/d CEE (Genant et al., 1997) or 25 µg 17β-estradiol (Samsioe et al., 2007) for one or two years, while in the cross-sectional trial by Cushing et al., 1998, postmenopausal women who were both current users of CEE and users of more than 8 years in duration were at higher risk for endometrial cancer.

PCNA is a protein synthesized in early G1 and S phases of the cell cycle and participates in the progression of the cell cycle. PCNA is a useful marker for detection of proliferating cells and can provide a prognostic index for endometrial cancer (Simionescu et al., 2006). It is also a sensitive measure of the effect of estrogen on the endometrium. Thus, the observation that cell proliferation between FS, LD, FS+LD and NEG groups did not differ from each other suggests that FS and LD do not exert estrogenic effects on uterine hyperplasia either alone or in
combination. However, morphological observations in the current study demonstrated an estrogen-like effect on the structure of the luminal epithelial cells in animals treated with FS, LD and FS+LD compared to the NEG group. Since both epithelial structure and cell proliferation in the uterus are sensitive markers of estrogenicity, these results suggest that FS, LD and FS+LD modulate some signs of estrogenicity (epithelial cell structure) and not others (PCNA), but the reason for these requires further investigation.

In conclusion, 10% FS, alone and in combination with LD, does not affect uterine wet weight and cell proliferation at the luminal epithelium in the ovariectomized rat model of postmenopausal osteoporosis. The observation that FS, alone and combined with LD induces similar estrogen-like effects on the morphology of the luminal epithelium that were lower in magnitude to the POS group suggest that FS does not modulate the effect of LD on uterine morphology however, further investigation is warranted to confirm their potential action on modulating carcinoma risk.
Chapter 9
General Discussion
9.0 General Discussion

9.1 General Discussion

The overall objective of this research was to determine the effects and mechanisms of FS, alone or in combination with lower doses of estrogen therapy, on deterioration of bone tissue and uterus health in the ovariectomized rat model of postmenopausal osteoporosis. The inclusion of a comprehensive set of outcome measures such as BMD, three-dimensional bone structure, and biomechanical bone strength offered a robust analysis of treatment-induced effects on skeletal health. In addition, the determination of whether n3 PUFA or lignans are accessible to skeletal tissue provided the opportunity to determine whether multiple components of FS may have the potential to directly modulate bone metabolism in the ovariectomized rat model. Moreover, biochemical and histological analyses of markers of bone turnover as well as histological analyses of uterine tissue of ovariectomized rats fed FS alone or combined with LD permitted for a more detailed examination into how treatment-induced effects may alter skeletal health and whether they can exert side-effects in a tissue of interest related to transdermal estrogen monotherapy.

In ovariectomized rats, FS was neither protective nor detrimental to bone quantity, bone quality or bone strength while low-dose estrogen therapy (LD) produced mild benefits to strength at the lumbar vertebrae (LV) (Study 1, Chapter 4) (Sacco et al., 2009b). The greatest protection to BMD, structure and strength at the LV occurred when FS was combined with LD. Moreover, the greatest benefits to bone occurred at the LV and not at the femurs or tibias (Study 1, Chapter 4) (Sacco et al., 2009b). FS+ULD also protected against ovariectomy-induced loss of BMD and strength at the LV however, these effects were intermediate to both the positive and NEG groups (Study 2, Chapter 5) (Sacco et al., 2009a). FS was also shown to not antagonize
ULD on bone. Study 1 and Study 2 both demonstrated that the LV and tibias of ovariectomized rats are responsive to dietary n-3 PUFA as FS feeding resulted in significantly higher relative levels of ALA, EPA and/or DHA in the skeletal tissue. Moreover, EPA was significantly higher in the LV of FS+LD group versus the FS group. The LV and long bones were also responsive to SDG ingestion as shown in Study 3 (Chapter 6) (Sacco et al., In Press), whereby ovariectomized rats gavaged daily with $^3$H-SDG resulted in the detection of radioactivity in skeletal tissue. Thus, both the lignans and n-3-PUFA may play a direct role in protecting against deterioration of vertebral tissue when FS is combined with LD. Mechanisms whereby FS+LD result in the greatest protection against deterioration of bone tissue at the LV were shown to be in part due to a decrease in bone turnover as depicted by lower osteocalcin levels in the serum and LV (Study 4, Chapter 7). FS+LD also resulted in higher OPG compared to the NEG while the effects induced by LD were intermediate to both the FS+LD and NEG groups. In Study 5 (Chapter 8), no differences in uterine weight or cell proliferation were detected between treatment groups. Thus, FS+LD results in the greatest protection against deterioration of bone tissue in part due to a decrease in bone formation and bone turnover (Study 4, Chapter 7). Moreover, FS+LD does not modulate the effect of LD on uterine weight and cell proliferation (Study 5, Chapter 8).

While FS alone was neither beneficial nor detrimental to BMD, three-dimensional structure and biomechanical bone strength in ovariectomized rats, it provided an additive benefit to LD on vertebral BMD, structure and strength (Figure 9-1) (Sacco et al., 2009b). In Study 2, FS did not provide an additive benefit to ULD on BMD and strength at the LV, femurs and tibias (Sacco et al., 2009a). These findings suggest that FS provides the greatest protection against
Figure 9-1: Summary of the effects and mechanisms of flaxseed (FS), alone or combined with lower doses of estrogen therapy, on deterioration of bone tissue and uterus health in the ovariectomized rat model of postmenopausal osteoporosis. Results from each group are compared to the negative control (NEG) and are depicted with various shades (FS= white, low-dose (LD) or ultra-low-dose (ULD) estrogen therapy = blue,
FS+LD or FS+ULD= black). Two arrows ⬇️↑️ depict statistical significance versus the NEG, one arrow ⬇️ denotes an intermediate effect and □️ denotes no effect. N/A= not applicable
ovariectomy-induced bone loss when combined with LD and it does not antagonize very low doses of estrogen therapy (i.e. ULD). These results are relevant to postmenopausal women who combine dietary bioactives (e.g. FS) with pharmacologic agents (e.g. HT) to attenuate postmenopausal bone loss.

The observation that the greatest treatment-induced effects on BMD and strength occurred at the LV compared to the femurs and tibias is expected since trabecular bone is more metabolically active than cortical bone (Bell et al., 1967; Hadjidakis & Androulakis, 2006) and thus, it is more likely to respond to FS and LD treatments. The greater treatment-induced effects observed at the LV versus long bones may have important implications if replicated in the clinical setting, since the target population of HT is women in the early menopause, which are more susceptible to vertebral fractures compared to fractures at any other site (e.g. hip, wrist). Moreover, since LD is not as effective in preserving bone mass as standard dose HT in the clinical setting, the findings from the present research suggest that a lower dose of estrogen may be used while maintaining its efficacy because FS offers extra protection against deterioration of bone at the LV when combined with LD.

The observation that FS+LD exerted the greatest protection to BMD, bone structure and strength in ovariectomized rats may be due to the potential interaction of the major components of FS (ALA, SDG) with LD. Indeed, this hypothesis is biologically plausible since Study 1, Study 2, and Study 3 demonstrated that ALA, EPA, DHA as well as SDG metabolites are accessible to skeletal tissue in ovariectomized rats after FS feeding, which is consistent with others that have also demonstrated that various tissues of rats are responsive to feeding FS oil or tritium-labeled SDG (Rickard & Thompson, 1998; Li et al., 2003; Reinwald et al., 2004; Saarinen et al., 2008; Lau et al., 2010; Saarinen & Thompson, 2010). Thus, ALA and SDG may
exert direct action on bone cells to modulate BMD, structure and strength when combined with LD. It is difficult to speculate whether ALA and its metabolites or the mammalian lignans may be responsible for the observed protection to BMD, structure and strength at the LV when FS was combined with LD for several reasons. Study 1 (Chapter 4) (Sacco et al., 2009b) demonstrated that FS and FS+LD treatments resulted in significantly higher ALA, EPA and DHA at the LV compared to the NEG group and, EPA was significantly higher with FS+LD compared to LD treatment. Although these observations do not confirm that the n-3 PUFA content of FS is responsible for the observed effects on bone when FS was combined with LD, they agree with previous research that has observed a protective effect on BMD and bone metabolism when EPA or DHA was combined with estrogen (Schlemmer et al., 1999; Poulsen et al., 2008). In addition, the ALA concentration in the FS used for the current research (64%) is higher than what has been previously reported by others (45%-60%) (Cunnane, 2003; Daun et al., 2003; USDA, 2009) Moreover, previous research using various animal and human models has demonstrated more positive effects on bone metabolism with the use of ALA-rich diets and FS oil (Weiss et al., 2005; Griel et al., 2007; Boulbaroud et al., 2008) than with lignans (Kardinaal et al., 1998; Ward et al., 2001b; Kim et al., 2002; Power et al., 2006). Indeed, while the SDG concentration in the FS used for the current research (0.34%) is within the range of SDG concentrations previously reported (0.165%-0.375%) (Milder et al., 2005; Penalvo et al., 2005; Thompson et al., 2006; Smeds et al., 2007; Peterson et al., 2010), Study 3 (Chapter 6) (Sacco et al., In Press) showed that the incorporation of lignan metabolites into the LV was low (0.1%) following a daily gavage of $^3$H-SDG while being fed a 10% FS diet for one week. However, the amount of radioactivity recovered in the vertebra ($0.64 \pm 0.18$ pmol/g tissue) is half of peak circulating 17β-estradiol levels in rats ($0.128$ pmol/mL) (Reinwald & Burr, 2008).
and this amount does not take into consideration the lignan levels achieved in the skeletal tissue from the FS diet in Study 3. Thus, it possible that both the ALA or SDG components were responsible for the observed protection to bone when FS was combined with LD however, this requires future investigation.

The observations in Study 1 (Chapter 4) (Sacco et al., 2009b), whereby no significant relationships between the relative levels of n-3 PUFA in LV and outcome measures of bone health, does not agree with the hypothesis that the ALA in FS can be responsible for the protective effects observed on LV when FS is combined with LD. It is possible that an interaction occurred between ALA or its metabolites with estrogen to induce skeletal protection as previously demonstrated by others (Schlemmer et al., 1999; Poulsen et al., 2008). Indeed, these studies demonstrated that combining long-chain PUFAs (gamma-linoleic acid with EPA, or DHA) with estrogen potentiated the effects of estrogen on skeletal calcium content, femur BMC, and markers of bone turnover (DPY, osteocalcin) and inflammation (IL-6) in ovariectomized rats. Potential mechanisms of action attributed to the combined effects of long-chain PUFAs with estrogen on preserving bone have been proposed to include the prevention of n-6 PUFA production of pro-inflammatory compounds such as PGE₂, as well as altering membrane fluidity and phospholipid composition which can alter cell signaling pathways (Schlemmer et al., 1999; Poulsen et al., 2008).

While the effect of estrogen depletion (e.g. ovariectomy in rats, menopause in humans) on skeletal physiology is an increase in bone turnover, particularly due to an increase in bone resorption, Study 4 (Chapter 7) demonstrated that FS+LD resulted in lower osteocalcin and higher OPG scores at the LV compared to the NEG group, while LD did not. Since osteocalcin is a marker of both bone formation and bone turnover (Polak-Jonkisz 1998), these results
indicate that there may be a small benefit to reducing bone turnover when FS is combined with LD. In contrast to the LV, LD and FS+LD similarly reduce markers of bone turnover in the serum, as observed by lower serum TRAP-5β, CTX and osteocalcin levels compared to NEG. It is possible that a shorter or longer study duration may have revealed greater differences in makers of bone turnover between the FS+LD and LD groups. Indeed, changes in bone turnover have been observed in both sera and coccyges of rats as early as one week upon ovariectomy or upon the initiation of antiresorptive therapies (Frolik et al., 1996; Miyazaki et al., 2004).

Moreover, changes in biochemical markers of bone turnover occur more rapidly than changes in BMD, structure and strength after the initiation of antiresorptive therapy in both rats and humans and, differences in bone volume fracture at the LV were already observed following 2 weeks of treatments in Study 4 (Chapter 7). Thus, it is possible that significant differences in markers of bone turnover between the FS+LD and LD groups occurred closer to the initiation of treatments (e.g. 1 week post-initiation of treatments).

Like skeletal tissue, the uterus is another estrogen-sensitive tissue in which compounds with a similar structure to estrogen can induce cell proliferation, subsequent growth (Power et al., 2006; Penttinen et al., 2007; Kieback et al., 2010; Pinkerton & Goldstein, 2010) and potentially increase the risk for the development of endometrial cancer (Braithwaite et al., 2003). Thus, the neutral effects of FS, LD and FS+LD on uterine weight and cell proliferation of the luminal epithelium suggests that FS alone, or combined with LD, may not increase endometrial cancer risk. These results agree with previous research that have investigated the effects of FS, FS hull, SDG, ED and EL at various levels (Chen et al., 2004; Power et al., 2006; Power et al., 2007; Chen et al., 2009). However, FS, LD and FS+LD induced estrogen-like effects on the morphology of the luminal epithelia (elongated cells that were columnar in shape) compared to
the NEG (ovariectomized control) group. Although these effects were lower in magnitude compared to the POS (sham control) group further investigation is required to determine whether FS, alone or combined with LD may modulate carcinoma risk in the endometrium.

The 10% level of dietary FS was chosen for all studies in the present research because it can be attained in the human diet and it represents an intake of 25-50 g or 2½-5 tablespoons of FS per day. In addition, a 10% dietary FS level exerts a number of health benefits on other tissues such as the breast, prostate and in the cardiovascular system and, it has been shown to reduce tumor growth, serum/plasma cholesterol, and atherosclerosis in animal and clinical models (Lucas et al., 2002; Dodin et al., 2005; Thompson et al., 2005; Bergman Jungestrom et al., 2007; Chen et al., 2007; Dupasquier et al., 2007; Lindahl et al., 2011). Moreover, ingestion of a 10% FS diet has previously been shown to produce large amounts of SDG metabolites in both rats and humans (Rickard et al., 1996; Nesbitt et al., 1999; Coulman et al., 2005; Liu et al., 2006; Coulman et al., 2009). Thus, a 10% level of FS was most logical for the current research.

9.2 Strengths and Limitations

The research in this thesis contains many strengths. The ovariectomized rat model for postmenopausal osteoporosis was utilized in the present research because it is a well established FDA-approved model for preclinical investigation of the effects of dietary and pharmacological compounds on BMD, bone structure and strength (Turner, 2001; Iwaniec & Turner, 2008). In addition, the ovariectomized rat models postmenopausal bone loss in humans and their response to various pharmacological treatments including estrogen therapy (U.S. Food and Drug Administration, 1994). The detection of adverse side effects from various treatments is also possible using ovariectomized rats (U.S. Food and Drug Administration, 1994). Moreover, rats
are able to convert SDG into mammalian lignans (Rickard et al., 1996; Liu et al., 2006). Other strengths of the present research are that multiple doses of transdermal estrogen monotherapy that are relevant to the clinical setting (North American Menopause Society, 2010b) were examined and, the amount of FS administered in the current study is an amount that can be achieved by diet alone. Another strength is that a comprehensive set of outcome measures that are indicative of both bone quantity and bone quality were measured and can be used as surrogate measures of fracture risk. According to the WHO, BMD is the standard for diagnosing osteoporosis (WHO, 1994) however, approximately half of all postmenopausal fractures occur in women with BMD scores that do not meet the diagnostic criteria of osteoporosis (Stone et al., 2003; Schuit et al., 2004). Thus, our approach was appropriate as it investigated many complimentary measures of bone health.

In addition to these strengths, there are limitations of this research. The exact compounds or their metabolic products that are responsible for attenuating bone loss when FS was combined with LD are not known. From Study 1 (Chapter 4) (Sacco et al., 2009b), it is unknown whether the 0.25% higher level of EPA in the LV from FS+LD treatment compared to FS alone is sufficient to induce a functional difference on skeletal health between groups. In addition, from Study 3 (Chapter 6) (Sacco et al., In Press), it is unknown whether the level of SDG metabolites that reached the skeletal tissue over the study duration (7.08 ug per gram of tissue) can also induce a functional difference in skeletal health between treatment groups. Moreover, it is unknown whether the amount of SDG metabolites accessible to the skeleton may have differed if rats were also treated with LD when gavaged with $^3$H-SDG while being fed FS. A treatment group consisting of rats with a LD implant that would have also been gavaged with $^3$H-SDG while being fed a 10% FS diet was not included in Study 3 because of the low availability and
high cost of $^3$H-SDG. Thus, it is unknown whether SDG metabolites may have played a role in inducing a functional effect on BMD, structure and strength of the LV when combined with LD in Study 1. Another limitation of the present research is that rats lack haversian systems that are found in human cortical bone (Turner, 2001). Since haversian systems are structural units within cortical bone whereby bone remodeling takes place, treatment-induced effects on cortical bone remodeling cannot be fully elucidated in the ovariectomized rat model. Whether FS+LD attenuates the loss of BMD, strength and deterioration of bone tissue through direct (e.g. ER-mediated pathways in bone cells) or indirect action (e.g. intestinal absorption of calcium, immunoregulation) on bone cell metabolism remains to be determined.

9.3 Future Research

The results of the present research are clinically relevant to younger postmenopausal women that use HT for treatment of postmenopausal-related conditions including bone loss because they are likely to consume dietary supplements, many of which also contain phytoestrogens (Mahady et al., 2003; Singh & Levine, 2006). To gain a better understanding of which major components of FS (lignans, n-3 PUFA) are responsible for the greatest protection against ovariectomy-induced bone loss, deterioration of bone tissue and strength when combined with LD, an interesting follow-up study would be to examine the effect LD combined with SDG or ALA on bone loss using the ovariectomized rat model of postmenopausal osteoporosis. Including a FS+LD group (such as in the present research) could provide further insight to determine whether a specific component of FS or its whole ground seed are responsible for the interaction with LD to protect against deterioration of bone tissue in ovariectomized rats.
In addition, future studies should determine which specific metabolites of SDG have the greatest availability to skeletal tissue. While validated GC-MS and HPLC methods could be implemented to determine urinary and plasma levels of SDG metabolites (SECO, ED, EL) (Rickard & Thompson, 2000; Luoto et al., 2010), no validated methods are available to determine skeletal levels of SDG metabolites. It may be possible then to develop SDG metabolites labeled with different isotopes which could then be gavaged in ovariectomized rats to determine the accessibility of the specific SDG metabolites in skeletal tissue.

The effects of FS combined with LD on skeletal health of ovariectomized rats were predominantly observed in the LV. To fully obtain a better reflection on how cortical bone can be affected by FS combined with LD, a future study could include structural analyses of cortical bones of ovariectomized rats using uCT. Since ovariectomized rats and postmenopausal women similarly experience endocortical bone loss and an increase in medullary area, uCT analyses could provide insight into treatment-induced effects on the structure of skeletal sites (e.g. tibia and femur midpoints) that are rich in cortical bone. These analyses could provide rationale into implemented experiments of longer duration, to observe whether long-term treatments with FS+LD could modulate cortical strength in the postmenopausal situation. Another study could utilize other animal models that contain a true Haversian system such as the mouse.

The mechanisms whereby FS combined with LD exert the greatest protection to BMD, bone structure and strength at the LV remain to be elucidated. Future studies could include quantitative immunohistochemical analyses on markers of bone formation (alkaline phosphatase, osteocalcin, type I collagen), bone resorption (CTX, NTX, DPY) and overall bone turnover (osteocalcin, CTX) using imaging software, which would provide a more sensitive analysis on bone sections compared to scoring methods using light microscopy, which was used in the
present research. Other future studies could investigate treatment-induced effects on bone turnover using dynamic histomorphometry, which can provide measurements such as bone formation rate and mineral apposition rate and greater insight on how FS combined with LD affects bone turnover and mineralization. In addition, other molecular biology techniques such as polymerase chain reaction (PCR) and western blotting can be implemented to examine how the combined treatment of FS and LD affect ER status and signaling pathways (growth factor pathways, MAPK pathway, WNT pathway) that are involved in modulating the production of markers of bone turnover (osterix, osteocalcin, CTX, etc.) and in regulating bone health.

In ovariectomized rats, FS combined with LD does not result in greater uterine weight or cell proliferation of the luminal epithelium compared to either treatment alone. These findings are noteworthy because lower doses of HT such as LD are being investigated for the treatment of postmenopausal-related conditions including bone loss using the transdermal delivery system because they may be associated with fewer side effects compared to the combined estrogen and progesterone HT formulation. Thus, LD may be prescribed to symptomatic postmenopausal women regardless of hysterectomy status. Future studies could determine whether FS combined with LD affects cell proliferation and structure of glandular epithelium, endometrial stroma and the myometrium since they are also implicated in endometrial carcinomas (Uharcek, 2008). A clinical study could be conducted whereby postmenopausal women using LD are randomized to receive daily 10% ground FS or placebo (e.g. wheat germ) for 2 years. BMD and bone structure closer to the midpoint of long bones could then be measured using peripheral quantitative computed tomography (pQCT) and, finite element analysis from pQCT measurements can be implemented to predict strength at cortical-rich areas of the skeleton. Since human studies have investigated the effect of LD monotherapy on bone loss in
postmenopausal women with an intact uterus, a future RCT testing the combined effects of FS and LD versus LD alone on endometrial hyperplasia could be conducted to evaluate endometrial response to FS combined with LD.
CHAPTER 10
Conclusions
10.0 Conclusions

Using the ovariectomized rat model of postmenopausal osteoporosis,

1. FS is neither beneficial nor detrimental to BMD, three-dimensional bone structure, and biomechanical bone strength. FS provides the greatest protection to BMD, three-dimensional bone structure, and biomechanical bone strength when combined with LD, and not ULD, compared to either treatment alone (Chapters 4 and 5).

2. FS feeding results in the incorporation of ALA and its longer-chain metabolites (EPA and/or DHA) as well as the incorporation of SDG metabolites in skeletal tissue (Chapters 4, 5 and 6).

3. The mechanisms whereby FS combined with LD provides the greatest protection to BMD, three-dimensional bone structure, and biomechanical bone strength is in part due to a decrease in bone turnover (osteocalcin) (Chapter 7).

4. FS, alone or combined with LD, induces mild estrogen-like effects on uterine morphology however, these effects are lower than the POS group. In addition FS, alone or combined with LD, does not affect uterine wet weight or cell proliferation of luminal epithelium in the uterus (Chapter 8).

Overall: 10% FS does not protect against deterioration of bone tissue. The greatest protection to bone occurs when FS is combined with LD, which is in part due to a decrease in bone turnover. Moreover, FS, alone and combined with LD, does not modulate uterine weight and cell proliferation.
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U.S. Food and Drug Administration (1994) Guidelines for preclinical and clinical evaluation of agents used in the prevention or treatment of postmenopausal osteoporosis


Appendix
Appendix

Contributions to Studies

Study 1 and Study 2: Sandra Sacco, Wendy Ward, David Ma and Lilian Thompson provided intellectual contribution to the study design. Sandra Sacco conducted the in vivo portion of the study including implanting estradiol pellets, and with the assistance of Jessica Jiang, coordinated the daily care of the rats which included weekly body weights and thrice weekly measurement of food intake. Sandra Sacco performed all bone analyses: BMD by DEXA, bone structure by micro-computed tomography and strength testing by biomechanical strength testing. With the assistance from Sandra Reza-Lôpe and Jessica Jiang, Sandra Sacco developed the method for fatty acid extraction of skeletal tissue and performed fatty acid analysis using gas chromatography. Sandra Sacco was responsible for data management, statistical analyses and wrote the first draft of submissions to Menopause and Journal of Environmental Health and Toxicology and was responsible for incorporating author feedback into these manuscripts. Wendy Ward assisted Sandra with the writing and revising of these manuscripts.

Study 3: Sandra Sacco, Wendy Ward, Lilian Thompson and Bernhard Ganss provided intellectual contribution to the study design. Sandra conducted the in vivo portion of the study including implanting gavaging of tritiun-labeled SDG, and coordinated the daily care of the rats which included weekly body. Sandra Sacco collected and processed the organs for analysis using scintillation counting. Sandra Sacco wrote the first draft of the submission to the Journal of Medicinal Food and was responsible for incorporating feedback from other authors into the manuscript. Wendy Ward assisted Sandra with the writing and revising of this manuscript.
**Study 4:** Sandra Sacco, Wendy Ward and Bernhard Ganss provided intellectual contribution to the study design. Sandra Sacco conducted the in vivo portion of the study including implanting estradiol pellets. Sandra Sacco and Jim Chen performed immunohistochemical staining of lumbar vertebra sections and Jim Chen performed the analyses of the immunohistochemical stains. Bernhard Ganss and Jim Chen provided intellectual contribution in analyzing and presenting immunohistological results. Submission of this paper to a peer-reviewed journal is in preparation.

**Study 5:** Sandra Sacco and Wendy Ward provided intellectual contribution to the uterine analyses for the uterine tissue obtained from Study 1. Jessica Jiang performed the paraffin-embedding, sectioning and staining of uterine sections with hemaxotylin and eosin staining. Sandra Sacco performed the PCNA staining and analyzed the uterine slides for morphological and cell proliferation indices. Submission of this paper to a peer-reviewed journal is in preparation.
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  Sacco SM, Jiang JM, Reza-Lopez S, Ma DW, Thompson LU & Ward WE (2009) Flaxseed combined with low-dose estrogen therapy preserves bone tissue in ovariectomized rats. *Menopause* 16, 545-554. (Chapter 4, Study 1)

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