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Flaxseed Enhances the Beneficial Effect of Low-Dose Estrogen Therapy at Reducing Bone Turnover and Preserving Bone Microarchitecture in Ovariectomized Rats

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Abstract:

Our previous research showed greatest protection to vertebral bone mineral density and strength in ovariectomized (OVX) rats when lignan- and alpha-linolenic acid-rich flaxseed (FS) is combined with low-dose estrogen therapy (LD) compared to either treatment alone. This study determined the effects of combined FS+LD on serum and tissue markers of bone turnover and microarchitecture to explain our previous findings. 3-month-old OVX rats were randomized to negative control (NEG), FS, LD or FS+LD for 2 or 12 weeks, meaningful time points for determining effects on markers of bone metabolism and bone structure, respectively. Ground FS was added to the AIN-93M diet (100g/kg diet) and LD (0.42 μ g 17 β -estradiol/kg body weight/day) was delivered by subcutaneous implant. Sham rats were included as positive control. Bone formation (e.g. osteocalcin), bone resorption (e.g. tartrate-resistant acid phosphatase-5 β (TRAP-5 β)), as well as osteoprotegerin (OPG) and receptor activator of nuclear factor kappa-B ligand (RANKL) were analyzed from the 2-week study by commercial assays (serum) and/or histology (vertebra). Vertebral bone microarchitecture was measured from the 12-week study using microcomputed tomography. In serum, FS+LD and LD induced lower TRAP-5 β and osteocalcin, and higher OPG and OPG/RANKL ratio versus NEG and FS ($p<0.05$). In vertebrae, FS+LD induced higher OPG and lower osteocalcin versus NEG ($p<0.01$) and did not differ from LD and FS. FS+LD improved bone microarchitecture versus NEG, FS and LD ($p<0.05$). In conclusion, FS+LD protects bone tissue due to a reduction in bone turnover. However, elucidating the distinctive action of FS+LD on bone turnover compared to LD requires further investigation.

Key Words: bone histology; bone microarchitecture; bone turnover; flaxseed; low-dose estrogen therapy; lumbar vertebra; ovariectomized rats

Introduction

Postmenopausal osteoporosis is a skeletal disease characterized by low bone mass and deterioration of bone tissue, predisposing an individual to an increased risk of fragility fracture (NIH Consensus Development Panel on osteoporosis Prevention, Diagnosis, and Therapy 2001). Fragility fractures are a significant burden to the Canadian health care system, and often result in chronic pain, decreased quality of life, loss of independence or even death (Osteoporosis Canada 2013). Due to concerns regarding adverse health effects of estrogen replacement therapy, there has been a significant drop in its use (Hersh et al. 2004) but interest in efficacy and safety of lower doses of estrogen therapy remains (North American Menopause Society 2012; Sacco et al. 2010). In particular, younger postmenopausal women may safely benefit from lower doses of estrogen therapy (North American Menopause Society 2012). Moreover, many postmenopausal women seek complementary approaches such as dietary supplements that often contain phytoestrogens, to manage postmenopausal-related conditions including bone loss (Mahady et al. 2003; Singh and Levine 2006). Thus, whether these dietary supplements, alone or in combination with estrogen therapy, provide an effective and safer treatment for postmenopausal bone loss is an area of interest.

Flaxseed (FS) is an oilseed commonly consumed in the Canadian diet and its production in Canada accounts for 40% of global production (Laux 2012). FS is rich in the phytoestrogen lignan secoisolariciresinol diglycoside (SDG), and in the n-3 polyunsaturated fatty acid (n-3 PUFA) α -linolenic acid (ALA), compounds that have been suggested to augment bone health (Griel et al. 2007; Lukas et al. 2011; Ward et al. 2001a; Ward and Thompson 2013) and to prevent or attenuate progression of major chronic diseases affecting Canadians: cardiovascular disease, breast cancer and diabetes (Hutchins et al. 2013; Lowcock et al. 2013; Pan et al. 2009).

93 When FS is consumed, its lignan metabolites can elicit weak estrogenic/antiestrogenic action in
94 hormone-sensitive tissues (Penttinen et al. 2007; Penttinen-Damdimopoulou et al. 2009) while
95 ALA and its metabolites attenuate the expression of cytokines (e.g. receptor activator of nuclear
96 factor kappa-B ligand (RANKL), tumor necrosis factor- α) (Casado-Diaz et al. 2012; Cohen et al.
97 2005) that are involved in stimulating osteoclastogenesis, bone resorption and subsequent bone
98 loss (Boyce and Xing 2007). Since postmenopausal bone loss is linked to low levels of
99 circulating 17 β -estradiol and increased production of proinflammatory cytokines, there is
100 biological plausibility that through its components, FS may exert protective action against
101 postmenopausal bone loss as well as increase the effectiveness of estrogen replacement therapy.
102 However, intervention with FS alone did not protect areal bone mineral density (aBMD) in
103 healthy postmenopausal women (Brooks et al. 2004; Dodin et al. 2005; Lucas et al. 2002) or
104 bone strength in ovariectomized (OVX) rats (Sacco et al. 2009), the established preclinical
105 model of postmenopausal osteoporosis (Thompson et al. 1995). A 10% FS diet has been shown
106 to enhance the effect of low-dose estrogen therapy (LD) on bone health in OVX rats (Sacco et al.
107 2009) by preventing the loss of aBMD and strength at the lumbar vertebrae (LV), but not in long
108 bones, compared to either treatment alone (Sacco et al. 2009). Thus, the present study
109 determined if alterations in markers of osteoblast and osteoclast activity in serum and LV
110 explain, at least in part, our previous finding that FS combined with LD exerted the greatest
111 protective effect on the BMD and strength of the LV (Sacco et al. 2009). Specifically, we
112 investigated the effect of 2 weeks treatment with FS+LD on bone turnover markers in serum and
113 at the local tissue level of the LV trabeculae. A two-week study duration was chosen because
114 treatment-induced effects on markers of bone turnover occur rapidly in rat blood and skeletal
115 tissue (Wronski et al. 1988; Zhang et al. 2007) and may explain the differences observed in

aBMD and strength after chronic treatments (12 weeks) in our previous study (Sacco et al. 2009). To complement these findings we also included structural analysis of LV – surrogate measure of bone fragility - from a longer-term study (12 weeks) that used the same interventions (Sacco et al. 2009).

Materials and Methods

Animals

Animal care and use conformed to the Guide to the Care and Use of Experimental Animals (Canadian Council on Animal Care 1993), and the experimental protocols were approved by the Animal Ethics Committee at the University of Toronto, Canada. Three-month-old OVX (n=56) and sham (POS, n=14) Sprague-Dawley rats were purchased from Charles River Canada (St-Constant, QC, Canada), housed two per cage and were maintained with a 12:12-hr light:dark cycle at 22°–24°C and 50% humidity.

Diets

The basal and FS diets were based on the AIN-93M (Reeves 1997) formulation. The FS diet consisted of the basal diet (BD) supplemented with 10% freshly ground FS (Johnson Seeds Ltd, Arborg, MB, Canada) corrected for the contribution of FS to available carbohydrates, protein, fat and fiber such that the diets were isocaloric. Corrections to the FS diet were not performed based on the contributions of calcium, magnesium, potassium, phosphorus and vitamin D from FS since their levels were negligible when comparing the FS diet to the BD. The BD was devoid of phytoestrogens. Both BD and FS diets were prepared by Dyets Inc. (Bethlehem, PA, USA) and stored at 4°C.

Study 1 Experimental Design

OVX and sham 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 17 β -estradiol pellet (13 μ g, 90 day release; Innovative Research of America, Sarasota, FL, USA); and (4) FS+LD, fed the FS diet and implanted with a 17 β -estradiol pellet. The 10% FS level was chosen for the present study because it is attainable in the human diet, representing an intake of 25-50 g or 2½-5 tablespoons of FS per day. Moreover, 10% FS represents a level that has been shown to reduce tumor growth, serum or plasma cholesterol, and atherosclerosis in animal and clinical models (Chen et al. 2007; Dupasquier et al. 2007; Lindahl et al. 2011; Lucas et al. 2002; Thompson et al. 2005). The sham-operated group was fed the BD and was included as a positive control group (POS). The pellets delivered 0.42 μ g 17 β -estradiol/kg body weight/day which mimics LD estrogen therapy (25 μ g/day transdermal estrogen therapy) currently prescribed to postmenopausal women (North American Menopause Society 2012), 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. Fresh diet was provided bi-weekly. Diets were consumed ad libitum since a previous study showed that ad libitum and pair feeding resulted in similar effects to bone health in ovariectomized rats of a similar age as the present study (Jiang et al. 2008). After two weeks of treatment, rats were euthanized using 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; Zhang et al. 2007) and may explain

the differences observed in aBMD by dual energy x-ray absorptiometry (DEXA) and strength after chronic treatments (Sacco et al. 2009). Blood was obtained by cardiac puncture and serum aliquots were stored at -80°C. LV5 were removed, cleaned of soft tissue, fixed in 10% buffered formalin for 24 hours, then stored at -20°C in 100% ethanol for histochemical analyses.

Study 2 Experimental Design

This study was as described previously (Sacco et al. 2009). Briefly, OVX (n=48) and sham rats (POS, n=12) were acclimatized for one week on the BD, after which the rats were randomized into the same groups as described for Study 1 except they received the interventions for 12 instead of 2 weeks. Food intake was measured daily and body weights were recorded weekly. Total food intake and body weight are reported in Sacco et al (2009). LV5 were removed, cleaned of soft tissue, and stored at -80°C until scanning of three-dimensional trabecular bone microarchitecture using microcomputed tomography. In contrast to LV, structure of femur and tibia was not determined since both strength and aBMD values were similar among all OVX groups in our previous results (Sacco et al. 2009). The lack of response of these long bones may be due to their greater proportion of cortical bone that is less metabolically active compared to trabecular bone that predominates in LV. aBMD and bone strength of femurs, tibias and lumbar vertebra were previously published (Sacco et al. 2009).

Serum Markers of Bone Turnover

To assess markers of bone resorption, serum levels of tartrate-resistant acid phosphatase-5 β (TRAP-5 β), C-terminal telopeptides of type I collagen (CTX), osteoprotegerin (OPG) and RANKL were measured. RANKL is a cytokine that stimulates osteoclast formation, activity and

survival while the cytokine OPG acts as a decoy receptor for RANKL (Khosla 2001; Leibbrandt and Penninger 2008). Since the ratio of OPG to RANKL is a major determinant of osteoclast formation, activation and survival (Horwood et al. 1998; Leibbrandt and Penninger 2008), it is often measured to assess its relationship to bone resorption in response to treatment. Enzyme-linked immunosorbent assay (ELISA) was used to measure serum TRAP-5 β (RatTRAP™) and CTX (RatLaps™, Immunodiagnostic Systems Inc, Fountain Hills, AZ, USA) according to the manufacturer's instructions. The lowest limits of detection for TRAP-5 β and CTX were 0.1 U/L and 2.0 ng/mL, respectively. The intra-assay coefficients of variation for TRAP-5 β and CTX were 0.30-9.12% and 1.74-9.21%, respectively. The inter-assay coefficients of variation for TRAP-5 β and CTX were 0.50-5.96% and 0.13-4.17%, respectively.

For markers of bone formation, serum osteocalcin was analyzed using one Rat Bone Panel (Millipore, Billerica, MA, USA) kit with Luminex™ multiplex reagents according to the manufacturer's instructions. 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 0.30-0.87%.

To assess the OPG-RANKL-RANK axis, serum levels of OPG and RANKL were measured using two Rat OPG-Single Plex and two Rat RANKL-Single Plex kits from Millipore with Luminex™ multiplex reagents according to the manufacturer's instructions. Data were collected using Luminex 100. 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 then determined based on serum levels of OPG and RANKL for each rat.

208

209 **Morphological and Histochemical Evaluation of Bone Turnover in Lumbar Vertebrae**

210 Morphological evaluation of LV5 was performed on hematoxylin and eosin stained sections.

211 Markers of bone resorption (TRAP-5 β , OPG, RANKL) and bone formation (osterix, osteocalcin)

212 in LV5 were evaluated by enzymatic or immunohistochemical staining. Decalcification in 10%

213 EDTA solution, paraffin-embedding, sectioning (5 μ m per section), hematoxylin and eosin and

214 enzyme histochemistry for TRAP-5 β staining of formalin-fixed LV were performed at the Centre

215 for Bone and Periodontal Research, McGill University, Montreal, QC, Canada, as previously

216 described (Nguyen-Yamamoto et al. 2010). For OPG, RANKL, osterix and osteocalcin staining,

217 5 μ m sections were deparaffinized, rehydrated and endogenous peroxidase was blocked with

218 aqueous 3% H₂O₂. The antigen was retrieved by heating in Tris-EDTA buffer at pH 8.0 for 20

219 minutes in a microwave oven. The primary antibodies were diluted in Diluent Buffer (Santa Cruz

220 Biotechnology, Santa Cruz, CA, USA), made of 0.05 M Tris-HCl buffer containing 0.1% Tween,

221 0.015 M sodium azide and stabilizing proteins to reduce background. Sections were incubated

222 overnight at 4°C with goat-polyclonal anti-OPG (sc-8468, Santa Cruz Biotechnology), goat-

223 polyclonal anti-RANKL (sc-7627, Santa Cruz Biotechnology), rabbit-polyclonal anti-Sp7/osterix

224 (ab22552, Abcam, Cambridge, MA, USA), and rabbit-polyclonal anti-osteocalcin (sc-30044,

225 Santa Cruz Biotechnology). After washing, slides were incubated with donkey anti-goat (sc-

226 2042) or goat anti-rabbit (sc-2040, Santa Cruz Biotechnology) secondary antibodies.

227 Streptavidin-HRP and AEC substrate chromagen were used to demonstrate the antigens. Control

228 sections were stained with antibody diluent instead of the primary antibodies, and processed in

229 the same manner as all other samples. All agents were obtained from Dako (Mississauga, ON,

230 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-5 β 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). The individual performing the microscopic evaluations was blinded to group assignment of the rats.

Microcomputed Tomography

Intact vertebrae (LV5) from each rat were placed in water and scanned in air at room temperature using microcomputed tomographical measurement (Scanco Medical, Basserdorft, Switzerland). The x-ray source was set at a tube voltage of 70 kVp and at a tube current of 114 μ A. High resolution mode with an X, Y and Z resolution of 6 μ m was used to scan the LV samples. Acquisition files were obtained at 1000 projections with 2048 samples each (per 180° rotation), 0.18° 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 (200 slices above and 200 slices below the midpoint) after the total height of the LV between the cranial and caudal plates of the bone were measured. The grey-scale threshold was set to 280 to separate bone from the surrounding tissue. 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).

Statistical Analyses

Statistical analyses were performed to detect differences among groups for serum (TRAP-5β, CTX, osteocalcin, OPG, RANKL, OPG/RANKL ratio) and lumbar vertebrae (expression of TRAP5β, osterix, osteocalcin, OPG, RANKL, OPG/RANKL ratio) outcomes as well as bone microarchitecture of 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 $p < 0.05$.

Results

STUDY 1

Serum Markers of Bone Turnover at 2 weeks Post-Ovariectomy

TRAP-5 β : FS+LD and LD resulted in lower TRAP-5 β levels than the NEG, FS and POS groups (p<0.05) (Figure 1A). NEG and FS had significantly lower TRAP-5 β levels compared to the POS group (p<0.001) and NEG and FS did not differ from each other (Figure 1A).

CTX: FS+LD and LD resulted in lower CTX levels than both the NEG and FS groups (p<0.001) (Figure 1B) and were similar to the POS group. FS and NEG groups had significantly higher CTX levels compared to the POS group (p<0.001) and NEG and FS did not differ from each other (Figure 1B).

Osteocalcin: FS+LD and LD resulted in lower osteocalcin levels than both the NEG and FS groups (p<0.01) and these levels were similar to the POS group (Figure 1C). FS and NEG groups had significantly higher Osteocalcin levels compared to the POS group (p<0.01) and NEG and FS did not differ from each other (Figure 1C).

OPG: FS+LD and LD resulted in the highest OPG levels among OVX groups (p<0.05), and these levels did not differ to the POS group (Figure 1D). FS resulted in higher serum OPG levels compared to NEG (p<0.05) while OPG was lower in the NEG compared to the POS group (p<0.05) (Figure 1D).

RANKL: FS+LD resulted in RANKL levels that were lower than the NEG and FS groups (p<0.05) and did not differ from the POS group (Figure 1E). NEG and FS groups had higher RANKL compared to the POS group (p<0.05) and NEG and FS did not differ from each other.

LD resulted in the lowest serum RANKL levels compared to all other groups ($p<0.05$) (Figure 1E).

OPG/RANKL Ratio: FS+LD and LD resulted in the highest OPG to RANKL ratios among OVX groups ($p<0.05$) and these ratios were similar to the POS group (Figure 1F). NEG and FS groups had lower ratio of OPG to RANKL compared to the POS group ($p<0.05$) and NEG and FS did not differ from each other (Figure 1F).

Morphological and Histochemical Evaluation of Bone Turnover at the Lumbar Vertebrae at 2 Weeks Post-Ovariectomy

Morphology of LV5: Phenotypical differences in trabecular properties of LV5 were visualized semi-quantitatively (Figure 2A) and qualitatively (Figure 2B) using hematoxylin and eosin staining. FS+LD resulted in a higher bone volume fraction ($p<0.05$) than the NEG and FS groups and did not differ from the LD group (Figure 2A). The POS group had a higher ($p<0.05$) bone volume fraction compared to all other treatment groups (Figure 2A). FS+LD treatment resulted in trabecular phenotypes that mostly closely resembled the POS group (Figure 2B). FS and LD groups exhibited LV5 structures that were intermediate to the POS and NEG groups in terms of trabecular number and separation. The NEG group demonstrated evidence of OVX-induced bone loss compared to the POS group: lower trabecular bone volume and fewer trabeculae (Figure 2B). Moreover, the separation between trabeculae appeared to be greater in the NEG group compared to the POS group (Figure 2B).

TRAP-5 β : There was a trend for FS+LD to have higher TRAP-5 β than NEG ($p=0.08$) (Figure 3A). Moreover, FS+LD and LD had similar expression of TRAP-5 β which also did not differ compared to the POS group, while FS was similar to the NEG group (Figure 3A). Greater staining for TRAP-5 β was observed in the POS group compared to the NEG group ($p<0.01$) (Figure 3A, Figure 3B).

Osterix: FS+LD, FS, and LD resulted in higher osteoblast expression of osterix compared to the NEG group ($p<0.05$), and did not differ from the POS group (Figure 3C). Osteoblast expression of osterix was higher in POS compared to the NEG group ($p<0.05$) (Figure 3C, Figure 3D).

Osteocalcin: FS+LD resulted in lower osteocalcin expression compared to the NEG group ($p<0.05$) and did not differ from the POS, FS or LD groups (Figure 4A). Osteoblast expression of osteocalcin was lower in the POS compared to the NEG group ($p<0.05$) (Figure 4A, Figure 4B).

OPG: FS+LD resulted in higher OPG expression compared to the NEG group ($p<0.001$) and did not differ from the POS, FS and LD groups (Figure 4C). Osteoblast expression of OPG in LV5 was higher in the POS compared to the NEG group ($p<0.001$) (Figure 4C, Figure 4D).

RANKL: RANKL expression in osteoblasts was similar among all OVX groups (Figure 4E). RANKL expression was significantly lower in the POS compared to the NEG group ($p<0.05$) (Figure 4E, Figure 4F).

STUDY 2

Bone Microarchitecture at 12 Weeks Post-Ovariectomy

The trabecular microarchitecture of LV5 was evaluated at the end of the twelve week study (Figure 5A). FS+LD resulted in higher BV/TV compared to both the NEG ($p<0.05$) and FS ($p<0.01$) groups and there was a trend for FS+LD to be higher than the LD group ($p=0.06$) (Figure 5B). The POS group resulted in the highest BV/TV compared to all other groups ($p<0.05$), while no other differences were observed (Figure 5B). FS+LD resulted in higher ($p<0.01$) Tb.N compared to the NEG, FS and LD groups and did not differ from the POS group while the LD group resulted in higher ($p<0.05$) Tb.N compared to the NEG and FS groups (Figure 5C). Like all other groups, FS+LD did not modulate Tb.Th (Figure 5D) however, FS+LD resulted in the lowest ($p<0.05$) Tb.Sp compared to all other OVX groups and was similar to the POS group (Figure 5E). The NEG, FS and LD groups resulted in higher ($p<0.01$) Tb.Sp compared to the POS group while no other differences were observed (Figure 5E).

Discussion

Intervention with FS+LD attenuated OVX-induced bone turnover. This was shown by the lower serum TRAP-5 β , CTX and osteocalcin compared to the NEG group that was OVX but received no intervention. The fact that the expression of TRAP-5 β , osterix and osteocalcin in LV was similar to the POS group suggests that combined intervention protects against OVX-induced bone loss and deterioration of bone tissue through modulation of bone cell numbers and bone cell activities. Together, these findings support the greater preservation of lumbar vertebra structure as observed in the present study, and our previous findings that lumbar vertebra BMD and strength is improved after 12 weeks of intervention (Sacco et al. 2009). That FS resulted in

serum levels of bone turnover markers that were less favorable and significantly different from LD and FS+LD emphasizes that the effect of FS+LD on bone turnover are due to LD and/or its combination with FS. Indeed, FS+LD preserved trabecular number and trabecular separation to a greater extent than LD alone indicating that FS enhances the protective effect of LD on trabecular microarchitecture in the spine. However, the observation that FS+LD did not differ from LD in some measures of bone turnover (e.g. TRAP-5 β , CTX, osteocalcin) suggests other markers of bone turnover may distinguish how FS+LD modulates bone formation or resorption to result in greater protection to microarchitecture of LV compared to LD.

Effects of FS combined with HRT on biochemical markers of bone metabolism have not been previously reported. Likewise, the effect of FS alone on bone turnover has not been thoroughly studied. In the few clinical and animal trials that have examined the effects of FS on bone turnover, mixed findings have been reported (Abdelkarem et al. 2011; Babu et al. 2000; Brooks et al. 2004, Farmer et al. 2007; Griel et al. 2007; Lucas et al. 2002; Ward et al. 2001a; Ward et al. 2001b). Our finding that FS alone did not impact serum markers of bone turnover is in agreement with most previous data reporting little or no effect on BMD, bone strength and bone turnover (Brooks et al. 2004; Dodin et al. 2005; Farmer et al. 2007; Lucas et al. 2002; Sacco et al. 2009). However, it is difficult to compare our current findings with these previous studies since they were conducted in various clinical populations (e.g., healthy postmenopausal women, middle-aged men) and animal models (e.g., growing mice and gilts), and they were of varying durations (6 weeks-12 months). Nevertheless, the present study demonstrated that a 10% FS diet results in higher serum levels of OPG with no differences in RANKL or the ratio of OPG to RANKL compared to the NEG group. Since the ratio of OPG to RANKL is a major determinant of osteoclast formation, activation and survival (Horwood et al. 1998; Leibbrandt

and Penninger 2008), the present findings provide further evidence that a 10% FS diet may not induce a physiologically significant impact on bone resorption in OVX rats.

Our observation that LD reduces serum biochemical markers of bone turnover including TRAP-5 β , CTX and osteocalcin agrees with established effects of estrogen therapy in OVX rats and in postmenopausal women (Delmas et al. 2004; Gopala et al. 2003; Huang et al. 2007; Mukherjee et al. 2006; Peris et al. 1999; Poulsen et al. 2008). In addition, the single observed difference in serum markers of bone turnover between the FS+LD and LD groups was that FS+LD resulted in significantly higher serum RANKL compared to the LD group. However, the ratio of OPG/RANKL did not differ between both LD and FS+LD groups. It is possible that the combined treatment of FS and LD improves bone formation but serum osteocalcin did not differ between the FS and FS+LD groups. Other serum markers of osteoblast development or activity (e.g. alkaline phosphatase, osteopontin, type I collagen) may distinguish the difference between the actions of FS+LD and LD on the vertebra in OVX rats. In addition, since differences in vertebral 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 as observed in previous work (Casari et al. 1997). Nevertheless, while serum biochemical markers of bone turnover are useful for the prediction of bone loss during the early stages post-OVX and for monitoring the effectiveness of antiresorptive treatments, they reflect bone turnover of the whole skeleton rather than specific skeletal sites (Seibel 2000). Thus, TRAP-5 β , osteocalcin, OPG and RANKL staining was also performed in LV as this skeletal site experienced the greatest protection to bone microarchitecture in the present study, as well as greater preservation of aBMD and strength in our previous study (Sacco et al. 2009). Protein expression of osterix was also determined through histochemical 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. This suggests that the enhanced protection to LV bone mass, structure and strength observed when FS is combined with LD compared to LD alone (Sacco et al. 2009) 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.

When comparing the changes in markers of bone metabolism in serum and LV, there were inconsistencies for some markers but consistency with others. These differences are likely due to differences in tissue-specific responses of different skeletal sites but may also be due to methodological differences. For example, FS resulted in higher serum OPG levels compared to NEG while no differences in OPG in LV 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 in LV.

While FS is rich in SDG and ALA, we cannot conclude what component(s) mediated the favourable effect. There are no data to directly support that SDG or its metabolites have estrogenic effects in bone (Penttinen et al. 2007; Ward et al. 2001b). Thus, rather than SDG, an interaction between the n-3 PUFA (i.e. ALA, or its metabolites, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) and LD may contribute to the stronger lumbar vertebrae compared to either treatment alone. Indeed, bone tissue is responsive to changes in dietary fat composition from FS feeding (Sacco et al. 2009). In addition, n-3 PUFA are associated with lower serum

levels of RANKL and TRAP-5 β as well as activation of NF- κ B and lower activity of COX-II, suggesting that n-3 PUFA may attenuate osteoclastogenesis (Rahman et al. 2009). It is possible that combining FS with LD prevents n-6 PUFA production of pro-inflammatory compounds and alters membrane fluidity and phospholipid composition which can alter cell signaling pathways (Nakamura et al. 2001; Poulsen et al. 2008; Schlemmer et al. 1999). Indeed, further investigation which combine FS lignans or n-3PUFA with LD is necessary to determine which components of FS induce the greatest protection to the lumbar vertebra of OVX rats when combined with LD.

In conclusion, FS combined with LD attenuates OVX-induced bone turnover as demonstrated both in the serum and in the LV, which may be in part due to its action on osteocalcin and the OPG-RANK-RANKL axis. The finding that FS combined with LD results in the greatest protection to LV bone microarchitecture complements our previous findings that showed the greatest preservation of aBMD and strength at the LV with this combination (Sacco et al. 2009). Our finding that markers of bone turnover did not differ between FS+LD and LD suggests that further investigation is required to determine how FS+LD alters osteoblast and osteoclast activity to result in a greater protection to bone mass, bone microarchitecture and bone strength at the LV.

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Figure Legends:

Figure 1: Serum levels of tartrate resistant acid phosphatase-5 β (TRAP-5 β) (A), C-terminal telopeptides of Type I collagen (CTX) (B), osteocalcin (C), osteoprotegerin (OPG) (D), receptor activator of nuclear factor κ β (RANKL) (E), and OPG/RANKL ratio (F) in ovariectomized rats. 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-5 β , 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 $\pm$ SEM. $n = 12-14$ rats/group (TRAP-5 β , CTX, OPG, RANKL, OPG/RANKL ratio), $n = 6-8$ rats/group (osteocalcin).

Figure 2: Effect of 10% dietary flaxseed (FS), low-dose estrogen therapy (LD) and their combination (FS+LD) on bone volume fraction (A) and bone morphology of the lumbar vertebra 5 (LV5) (B) in ovariectomized rats. The appearance of lower trabecular number and greater trabecular separation in NEG group compared to the POS group (depicted by arrows) (B) is characteristic of trabecular bone of rats after ovariectomy. 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. Data are expressed as mean $\pm$ SEM. $n = 8$ rats/group. 100x magnification.

Figure 3: Effect of 10% dietary flaxseed (FS), low-dose estrogen therapy (LD) and their combination (FS+LD) on the protein expression (A, C) and immunolocalization (B, D) of tartrate resistant acid phosphatase 5 β (TRAP-5 β) (A, B) and osterix (C, D) in lumbar

vertebra 5 (LV5) in ovariectomized rats. Arrows depict areas of positive staining for TRAP-5 β and 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 $\pm$ SEM. n= 7-8 rats/group. 400x magnification.

Figure 4: Effect of 10% dietary flaxseed (FS), low-dose estrogen therapy (LD) and their combination (FS+LD) on protein expression (A, C, E) and immunolocalization (B, D, F) of osteocalcin (A, B), osteoprotegerin (C, D) and receptor activator of nuclear factor $\kappa\beta$ (RANKL) (E, F) in lumbar vertebra 5 (LV5) (B) in ovariectomized rats. Arrows depict areas of positive staining for osteocalcin, OPG and RANKL. 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 for data that was normally distributed (osteocalcin, RANKL) or by Kruskal-Wallis one-way ANOVA followed by the Dunn's post-hoc test for data that was not normally distributed (OPG). Data are mean $\pm$ SEM. n= 7-8 rats/group. 400x magnification.

Figure 5: Effect of 10% dietary flaxseed (FS), low-dose estrogen therapy (LD) and their combination (FS+LD) on skeletal microarchitecture (representative images shown in Figure 5A), specifically bone volume fraction (BV/TV) (B), trabecular number (Tb.N) (C), trabecular thickness (Tb.Th) (D) and trabecular separation (Tb.Sp) (E) in lumbar vertebra 5 (LV5) in ovariectomized rats. Representative micro-computed tomography images (Figure 5A) of lumbar vertebra 4 (LV5) from each group demonstrate a visible deterioration of the trabecular network in the NEG and FS groups compared to the POS group. Bars with different

746 letters (a-c) indicate a significant difference among groups at $p < 0.05$ by one-way ANOVA
747 followed by Student-Newman Keul's post-hoc test Data are mean $\pm$ SEM. n= 12 rats/group.