MINIREVIEW

Long-Chain Polyunsaturated Fatty Acids and the Regulation of Bone Metabolism

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The role of prostaglandin E2 (PGE2) in the regulation of bone remodeling is well established. There is increasing evidence that various long-chain polyunsaturated fatty acids (LCPUFAs), as well as nonprostanoid LCPUFA metabolites, also have critical roles in regulating bone metabolism and may have therapeutic potential in the management of postmenopausal osteoporosis. Although only the 18-carbon precursors for the n-3 and n-6 LCPUFAs are deemed "dietary essential," the ability of the body to convert these precursor fatty acids into the more highly unsaturated 20- and 22-carbon LCPUFAs decreases with aging, menopause, and various lifestyle factors (e.g., smoking). Increasing dietary LCPUFA intake increases tissue and blood LCPUFA concentrations, as well as the concentrations of their metabolites. Modification of dietary LCPUFA content, particularly increasing the intake of n-3 LCPUFAs, has been shown to minimize the decline in bone mass caused by menopause in women and ovariectomy in animal models. This review summarizes findings from both *in vivo* and *in vitro* studies and outlines the effects of LCPUFAs and their metabolites on calcium balance, osteoblastogenesis, osteoclastogenesis, and osteoblast and osteoclast function. Exp Biol Med 232:1275–1288, 2007

Key words: bone; polyunsaturated fatty acids; lipid mediators

Introduction

Long-chain polyunsaturated fatty acids (LCPUFAs) and lipid mediators derived from LCPUFAs have critical roles in the regulation of a variety of biological processes. Although most renown for their role in the inflammatory process, lipid mediators are also involved in the regulation of stem cell proliferation and differentiation (1), cell cycle progression (2), and signal transduction (3). Their role in bone metabolism is summarized here.

Bone Metabolism

Bone is a dynamic organ. Although bone growth in humans largely ceases at the end of adolescence, bone is continually renewed or "remodeled" throughout adulthood, enabling the skeleton to adapt to the changing stresses of life. Bone remodeling involves the sequential and coupled resorption of a small area of bone tissue by specialized macrophage-like cells known as osteoclasts followed by replacement of this tissue with new bone synthesized by osteoblasts (4–6). In humans, an estimated 10% of bone is remodeled each year (7).

Bone remodeling is regulated by controlling osteoblast and osteoclast cell number and activity. Osteoclastogenesis is largely regulated by a triad of proteins consisting of a ligand, receptor-activated nuclear-kappa B ligand (RANK-L), its receptor, RANK, and a decoy receptor, osteoprotegerin (OPG). RANK is a membrane-bound protein expressed on osteoclast precursors and mature osteoclasts (8). RANK-L exists in both a membrane-bound and soluble form and is produced by a range of cells including osteoblasts and activated T cells (9). OPG is a soluble protein secreted by a range of different cells, including osteoblasts (8). Binding of RANK-L to RANK promotes osteoclastogenesis (10, 11) and inhibits osteoclast apoptosis

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(12). Binding of RANK-L to OPG prevents RANK-L/RANK-induced osteoclastogenesis, and increased OPG protein levels lead to a rapid reduction in osteoclast number (8). Hence, the balance between RANK, RANK-L, and OPG is a major factor controlling osteoclast number.

Various signaling pathways converge to regulate osteoblastogenesis. These include the canonical Wnt (13) and bone morphogenic protein (BMP) signaling pathways (14). Although several transcription factors are involved in osteoblastogenesis, core binding factor-1 (Cbfa-1, also known as RUNX2) and Osterix (Osx) have been identified as having particularly pivotal roles in controlling osteoblast differentiation (15, 16) and the activity of mature osteoblasts (17).

Bone remodeling occurs at discrete sites within the skeleton and is triggered in response to mechanical strain. Osteocytes are specialized "mechano-sensing" cells that reside within bone. They detect mechanical strain and initiate signaling pathways, leading to both osteoclastogenesis and osteoblastogenesis (18). Lipid mediators have a critical role in the mechano-signaling pathway. Within seconds of mechanical loading of bone, the lipid mediator prostaglandin E2 (PGE2) is released by osteocytes and mature osteoblasts (13). Phospholipase-mediated membrane release of fatty acids, notably arachidonic acid (AA, 20:4n-6), the substrate for PGE2 synthesis, and expression of the inducible form of cyclooxygenase (COX), COX-2, which oxidizes AA to PGE2, are upregulated as an early response to strain (19). PGE2 promotes osteoclastogenesis by stimulating expression of both RANK-L and RANK and inhibiting expression of OPG. PGE2 also activates the Wnt signaling pathway (13) and promotes *cbfa-1* (20, 21) and insulin-like growth factor 1 (IGF-1) (22) expression, thereby stimulating osteoblastogenesis.

Although the importance of AA and PGE2 in regulating bone remodeling is well established, the involvement of LCPUFAs in the control of bone metabolism may be much more extensive than is currently recognized. The role of prostaglandins in bone biology has been comprehensively reviewed elsewhere (23, 24). The present review focuses on the actions of LCPUFAs themselves on bone, as well as the effects of their nonprostanoid bioactive metabolites.

Long-Chain Polyunsaturated Fatty Acid Metabolism

LCPUFAs are fatty acids with a minimum chain length of 18 carbons containing at least 2 double bonds. LCPUFAs are classified into 1 of 2 families: n-3 and n-6. The n-3 and n-6 nomenclature refers to the location of the first unsaturated carbon from the methyl ('n') terminus of the fatty acid. The first double bond is located at carbon 3 for n-3 fatty acids and at carbon 6 for n-6 fatty acids.

Alpha-linolenic acid (ALA) (18:3) and linoleic acid (LA) (18:2) are the parent compounds for the n-3 and n-6 series of LCPUFAs, respectively. Because humans lack the ability to insert a double bond prior to carbon 9 in the fatty acid chain, ALA and LA cannot be synthesized endogenously and are therefore dietary essential fatty acids. The best dietary source of n-3 LCPUFAs is fish oil; however, ALA is present in plant chloroplasts, and therefore green leafy vegetables are also a source of n-3 fatty acids. n-6 LCPUFAs are present in many edible plant oils, such as corn and soybean (25), and are by far the most common LCPUFA in the typical Western diet. ALA and LA can be further elongated and desaturated by endogenous enzymes to form longer chain PUFAs.

LCPUFAs are precursors for a range of metabolites. The LCPUFA metabolites are oxidation products formed by the activities of cyclooxygenases (COX), lipoxygenases (LOX), cytochrome P450–like epoxygenases, and nonenzymatic oxidation. There are 2 broad categories of LCPUFA metabolites: eicosanoids and docosanoids. The eicosanoids are derived from the 20-carbon n-3 and n-6 LCPUFAs and include the prostaglandins, leukotrienes, thromboxanes, lipoxins, and E-series resolvins (26–29). Docosanoids are derived from the 22-carbon LCPUFAs. At present, only docosanoids stemming from the n-3 family have been identified. These are mono-, di-, and trihydroxylated derivatives of DHA and include the docosatrienes, protectins (also known as neuroprotectins), and D-series resolvins (26). A schematic diagram of LCPUFA metabolism is shown in Figure 1.

Cyclooxygenase. COX converts dihomogammalinolenic acid (DGLA), arachidonic acid (AA), and eicosapentaenoic acid (EPA) into prostaglandins of the 1-, 2-, and 3-series, respectively. COX also catalyzes the conversion of AA to thromboxane A2 (TxA2) (30) and, in conjunction with aspirin, the mono-hydroxylation of DHA to form 13R- and 17R-hydroxylated DHA (13R- and 17R-HDHA) (28). To date, 2 distinct *cox* genes have been identified encoding 2 isoforms of COX known as COX-1 and COX-2 (31). COX-1 is constitutively expressed in most tissues, whereas COX-2 is the inducible form of the enzyme. COX-1 and COX-2 have greater specificity for AA than EPA and therefore preferentially synthesize 2-series rather than 3-series prostaglandins (32). Due to the smaller size of the substrate binding site of COX-1 compared with COX-2, 22-carbon DHA can only be metabolized by COX-2 (28).

Members of the n-6 fatty acid family upregulate COX-2 expression and therefore promote 2-series prostaglandin formation. At least some members of the n-3 LCPUFA family inhibit COX-2 expression (33), possibly by modulating toll-like receptor signaling pathways (30).

The existence of a third isoform of COX has been hypothesized (31). However, although various alternative splice forms of both the *cox-1* and *cox-2* genes have been described, a third active isoform of COX has yet to be identified in humans (32, 33).

Lipoxygenase and Epoxygenase. There are several isoforms of the LOX enzyme that exhibit differing substrate specificities. 12/15-LOX activity results in generation of hydroperoxy derivatives of 20-carbon LCPUFAs.

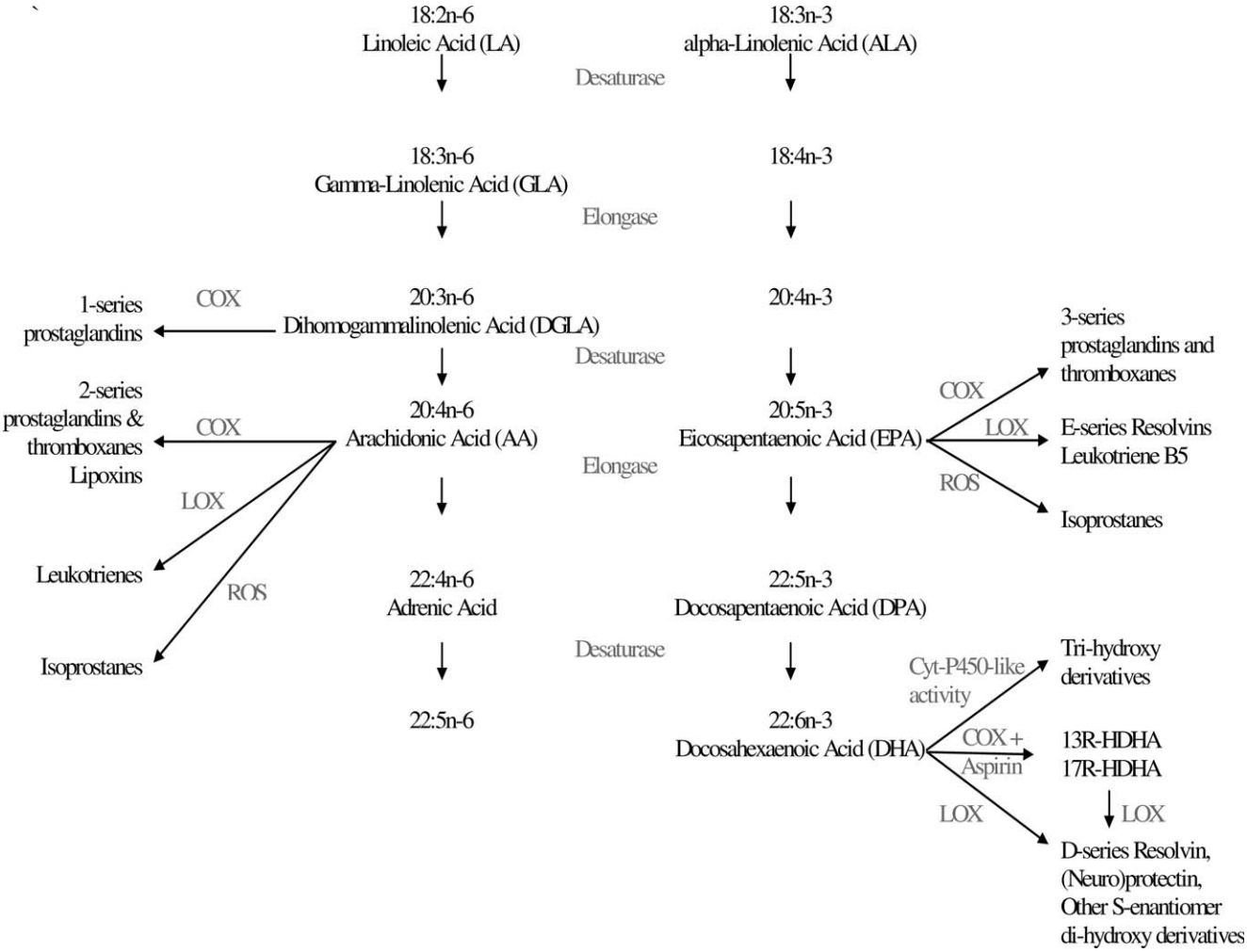

Figure 1. Metabolism of long-chain polyunsaturated fatty acids. Linoleic acid and alpha-linolenic acid are progressively desaturated and elongated by a shared desaturase/elongase enzyme system to form longer-chain and more highly unsaturated fatty acids. Diagram created from information in van Papendorp *et al.* (26), Serhan *et al.* (27), Serhan (28), Kuhn and O'Donnell (29), and Shen and Tai (30).

These can be subjected to further LOX activity, resulting in formation of leukotrienes, lipoxins, and hepoxilins (29). DHA is also a substrate for 15-LOX, resulting in generation of the mono- and dihydroxy DHA derivatives 17S-HDHA, 7S,17S-diHDHA, 10,17S-diHDHA ((Neuro)protectin D1), and 4S,17S-diHDHA. A combination of 15-LOX and 5-LOX epoxidase activity results in formation of further dihydroxy DHA metabolites, including 4S,17R-diHDHA, and 7S,17R-diHDHA (26, 28). LCPUFAs may also serve as substrates for cytochrome P450-catalyzed reactions as trihydroxy DHA derivatives are generated by cytochrome P450-like activity (26, 28).

Nonenzymatic Oxidation. Finally, nonenzymatic metabolism of LCPUFAs also occurs. The isoprostanes are highly oxidized LCPUFA metabolites formed by free-radical catalyzed oxidation of usually membrane-bound AA, EPA, and DHA (34).

Many of the LCPUFA metabolites generated either by enzymatic or nonenzymatic means have demonstrated bioactivity in mammalian systems.

Long-Chain Polyunsaturated Fatty Acids and Bone

Dietary LCPUFAs are incorporated into cell membranes within the body. The composition of LCPUFAs in the diet is reflected in the fatty acid composition of a variety of body tissues and fluids, including bone marrow; the periosteum (membrane surrounding long bones); bone (35); and red blood cell (RBC) membranes (36), serum (37), and plasma (39).

Dietary LCPUFA deficiency in animals and humans results in decreased intestinal calcium absorption (40), reduced synthesis of bone connective tissue matrix and loss of cartilage (39), bone demineralization (40), increased renal and arterial calcification (41), replacement of bone with adipose tissue (40), and severe osteoporosis (41). People who habitually consume a high-fish (high n-3 LCPUFA) diet, such as the Japanese and Greenland Eskimos, have a very low incidence of osteoporosis (40). Although a negative association between total LCPUFA intake and bone mineral density (BMD) was observed in one study in

postmenopausal women (42), a more recent study that examined dietary intake of the 2 families of LCPUFAs reported that postmenopausal women with a high dietary ratio of n-6:n-3 fatty acids had the lowest BMD (42). Therefore, high n-6 LCPUFA intake rather than high total LCPUFA intake may be detrimental to bone mass. In a longitudinal study in adolescent males, concentration of n-3 LCPUFAs in the phospholipid fraction of serum was positively correlated with change in total body and spine BMD (44). The association was greatest between serum phospholipid DHA concentration and BMD, which may indicate that specific LCPUFAs have anabolic effects on bone.

Intervention Studies: Human

Dietary intervention studies investigating the effect of LCPUFAs on bone health in postmenopausal women have yielded mixed results (Table 1). In elderly (mean age 80 years) osteoporotic women, daily supplementation with 4 g of fish oil containing 16% EPA and 11% DHA or a mixture of fish and evening primrose oils containing 60% linoleic acid, 8% GLA, 4% EPA, and 3% DHA for 16 weeks resulted in decreased serum alkaline phosphatase activity and increased serum concentration of procollagen. The combined evening primrose oil/fish oil supplement was also associated with a higher serum osteocalcin concentration compared with supplementation with olive oil, evening primrose oil, or fish oil alone (32). Osteocalcin is a bone-specific protein that is released into blood during both new matrix formation and osteoclastic breakdown of existing matrix. Circulating osteocalcin concentration is therefore indicative of the rate of bone turnover. Bone-specific alkaline phosphatase and procollagen are generally only released into the blood upon formation of new collagenous material (45) and are therefore biochemical markers of bone formation. The results from this study are therefore ambiguous because decreased serum alkaline phosphatase activity indicates a reduction in the rate of bone formation, whereas increased procollagen concentration suggests the opposite. Serum calcium concentration was slightly increased and urinary calcium clearance significantly increased in the fish oil supplemented group compared with all other groups (27). This may indicate increased bone resorption in this group and therefore signify a negative effect of fish oil on calcium balance. However, increased intestinal calcium absorption has been reported in other studies after the ingestion of n-3 fatty acids or fish oil (46, 47). Therefore, the increased urinary calcium excretion observed in elderly women after fish oil supplementation may be reflective of increased intestinal calcium absorption in the fish oil–supplemented group. Although these findings suggest that dietary supplementation with LCPUFAs can alter calcium balance and the rate of bone metabolism, whether this results in increased bone formation is less clear.

More conclusive evidence for a beneficial effect of LCPUFA supplementation on bone mass is provided by a second study in which elderly, osteoporotic or osteopenic women (mean age 79.5 years) with habitually low dietary calcium intakes were supplemented with 6 g of LCPUFA-rich oil (3.6 g LA, 480 mg GLA, 240 mg EPA, and 180 mg DHA) in conjunction with 600 mg of calcium carbonate per day for 18 months. Controls who received 600 mg of calcium carbonate and 6 g of coconut oil per day exhibited a 3.2% decrease in lumbar spine BMD over the 18-month period; however, BMD was maintained in the LCPUFA-supplemented group. Continuation of the LCPUFA/calcium supplementation for a further 18-month period resulted in an increase of 3.1% in lumbar spine BMD (39).

However, 2 subsequent studies showed no effect of LCPUFA supplementation on bone after menopause. In one study, pre- and postmenopausal women (age range 25–40 years and 50–65 years, respectively) were supplemented with Efacal, a Scotia Pharmaceuticals product containing a combination of evening primrose oil (4.0 g/day providing approximately 430 mg GLA/day), fish oil (440 mg/day providing approximately 70 mg EPA/day), and calcium (1.0 g/day) for a period of 12 months. No additional benefit of LCPUFA supplementation on total body BMD over calcium supplementation alone was observed (48). However, the changes in total body BMD over the 12-month study period were very small in both treatment groups (a decrease of 0.7% in the Efacal group and 0.9% in the calcium-supplemented group). Measurement of total body BMD may lack the sensitivity required for evaluating the effects of potential antiosteoporotic agents on bone over the relatively short study period. Generally, effects of antiosteoporotic treatments on bone mass are more apparent in sites rich in trabecular bone due to the higher rate of bone turnover in trabecular as opposed to cortical bone. Trabecular-rich bone sites, such as the femoral neck and lumbar spine, are also the most common sites of osteoporotic fracture (49).

In another study, menopausal women (age range 45–65 years) receiving 40 g of flaxseed oil supplement per day (a source of ALA but also of other bioactive components, such as lignans) for 12 months showed no significant difference in BMD at the end of the treatment period compared with women supplemented with a wheat germ placebo (50). The composition of LCPUFAs in this supplement differed considerably from the supplements used in the previous studies in that it contained only the 18-carbon n-3 LCPUFA rather than the longer-chain 20- and 22-carbon LCPUFAs. Observations from epidemiological studies show consumption of foods that are rich in EPA and DHA, such as fatty fish, is linked with positive effects on bone mass (43). Although ALA can be converted to EPA and DHA, this conversion is very inefficient. One study reported that only 6% of dietary ALA was converted to EPA and just 3.8% converted to DHA in humans consuming a high saturated fat diet (51). Consumption of an n-6 LCPUFA-rich diet appears to inhibit ALA elongation and desaturation because conversion of ALA to EPA and DHA was reduced by 40% to 50% when an n-6 LCPUFA-rich diet was consumed (51).

Table 1. Summary of Intervention Studies in Humans Examining the Role of Long-Chain Polyunsaturated Fatty Acids (LCPUFAs) on Bone Mass and Metabolism

Population characteristics	LCPUFA supplement	Study duration	Outcomes	Reference
Postmenopausal, osteoporotic women mean age 80 years	4 g/day evening primrose oil (EPO), fish oil (FO), or a combination of EPO + FO	16 weeks	Increased serum concentrations of procollagen, decreased serum alkaline phosphatase activity with FO or EPO + FO supplementation Increased serum osteocalcin with EPO + FO; increased urinary and serum calcium with FO	27
Premenopausal women aged 25–40 years at baseline Postmenopausal women aged 50–65 years at baseline	Efacal calcium 1.0 g Evening primrose oil 4 g Fish oil 440 mg per day	12 months	No effect of LCPUFA supplementation above that of calcium alone on total body bone mineral density or biochemical markers of bone formation in either pre- or postmenopausal women	52
Postmenopausal women aged 45–65 years	Flaxseed (contains α-linolenic acid as well as lignans) providing 16 g lipids from flaxseed (57% α-linolenic acid) per day	12 months	No effect of flaxseed on lumbar spine or femoral neck bone mineral density	54
Elderly postmenopausal women (mean age 79.5 years) with habitual low calcium intake	600 mg calcium 6 g LCPUFA-rich oil	36 months	Increased lumbar spine bone mineral density	43

Much higher concentrations of 20- and 22-carbon n-3 LCPUFAs were provided in the study reporting a positive effect of LCPUFA supplementation on bone mass than in the 2 trials reporting no effect on bone mass, which may mean that the very long-chain n-3 PUFAs have a beneficial effect on bone after menopause.

Intervention Studies: Animal

The vast majority of work in this field has been conducted in animals, although a range of different models have been used, including growing, growing-ovariectomized, mature-ovariectomized, and diabetic animals. A variety of different supplementation regimens have also been employed, the majority involving the use of combinations of LCPUFAs rather than individual fatty acids. Table 2 provides a summary of the intervention studies that have been conducted in animals.

Studies in Growing Animals. Male rats fed a LCPUFA-deficient diet during late gestation and lactation followed by a LCPUFA-sufficient diet exhibited significantly higher body weight and cortical bone mineral content (BMC), area, and thickness and significantly lower trabecular BMD compared with controls. Serum levels of IGF-1 and leptin were significantly lower in rats while on the LCPUFA-deficient diet but returned to normal levels once a LCPUFA-adequate diet was fed (52). Excess LCPUFA can also have a detrimental effect on bone mass. High-dose supplementation of either n-6 or n-3 LCPUFAs results in impaired bone formation during growth (52, 54). Supplementation of growing mice with flaxseed oil (a source of ALA) had no effect on bone mass or bone strength (55). However, dietary supplementation with longer chain, more highly desaturated LCPUFAs has been shown to influence bone mass. In piglets, low levels (0.6 g/100 g fat) of a supplement containing AA and DHA (5:1) increased bone mass; however, higher doses (1.2 g/100 g fat and 2.4 g/100 g fat) were less beneficial (56). Similarly, supplementation with AA (0.60%–0.75% total fat) and DHA (0.1% total fat) resulted in approximately 0.4% greater femur BMC in one study and approximately 10% greater whole body BMC compared with controls in one study in male piglets (57). In a second study, the same AA and DHA supplementation regimen was associated with 0.4% higher femur BMC compared with controls, whereas femur BMC was 4% greater than controls in animals receiving PGE2 by injection (58). However, combined treatment with AA, DHA, and PGE2 injection resulted in a 4.4% lower femur BMC compared with controls (58). This may be due to further elevation of PGE2 concentrations by synthesis from AA. PGE2 has a biphasic effect on bone. Low concentrations of PGE2 in conjunction with mechanical loading have an anabolic effect on bone mass (59). At high concentrations, PGE2 promotes bone resorption (60). In other studies, limiting PGE2 synthesis has been implicated as a means of optimizing bone mass. For instance, in 1-month old Japanese quail, n-3 LCPUFA supplementation

Table 2. Summary of Intervention Studies in Animals Examining the Role of Long-Chain Polyunsaturated Fatty Acids (LCPUFAs) on Bone Mass and Metabolism

Animal	LCPUFA supplement	LCPUFA amount
Growing intact animals		
Weanling male rats	Three ratios of GLA:EPA+DHA tested: 3:1, 1:1, and 1:3	% of GLA, EPA, and DHA in total dietary fat was 0.8% (3:1), 0.9% (1:1), and 1.4% (1:3)
Weanling male rabbits	Menhaden oil	10% of diet
Weanling male and female mice	Flaxseed oil	10% of diet
Weanling male piglets	AA:DHA 5.05:1	0.6%, 1.2%, or 6% of total dietary fat provided by AA and DHA
Weanling male piglets	Four ratios of AA:DHA tested: 3:1, 4.5:1, 6:1, and 7.5:1	0.4%, 0.6%, 0.7%, or 0.85% of total dietary fat provided by AA and DHA
Weanling male piglets	AA:DHA ratio 8:1 with and without PGE2 injection (0.1 mg/kg body weight/day)	0.9% of total dietary fat provided by AA and DHA
1-month-old Japanese quail	Menhaden oil	5% of diet
Weanling male rats	Four ratios of n-6:n-3 tested: 1.19:1, 2.6:1, 9.8:1, and 23.8:1	46.3%, 54.5%, 66.5%, or 70.5% of total dietary fat provided by LCPUFAs
Weanling male rats	n-6:n-3 ratio 1.4:1 fats provided by corn and menhaden oils	Menhaden oil 57% of total fat, corn oil 43% of total fat
Ovariectomized animals		
3-month-old ovariectomized rats	Two ratios of n-6:n-3 tested: 5:1 and 10:1	Each ratio tested in diets with a high total PUFA content and a low PUFA content. % LCPUFAs in high PUFA diet 67%–69% and in low PUFA diet, 38% of total fat
6-month-old ovariectomized rats	Low and high doses of EPA ethyl ester tested: 0.1 g and 1.0 g/kg rat body weight/day	4.0%–4.5% of total fat EPA for low dose and 40%–47.5% EPA in diet for high dose. Ratio of n-6;n-3 21:1 for low dose and 1.26:1 for high dose.
Mice 2 months old at trial commencement Ovariectomized at 4 months of age	Fish oil containing 14.3% EPA and 8.7% DHA	5% of diet was fish oil
17-week-old ovariectomized rats	EPA (free fatty acid) with either calcium-deficient or calcium-adequate diet	11% of total fat as EPA
2-month-old ovariectomized rats	Diets with 2 different DHA contents. n-6:n-3 ratio ~5:1 in both diets. Amount of DHA and EPA in diets not stated.	n-3 LCPUFAs 10.29% (low DHA) and 11.39% (high DHA) of total fat. n-6 LCPUFAs 53.05% (low DHA) and 55.54% (high DHA) of total fat
3-month-old ovariectomized rats	Four ratios of GLA:EPA+DHA tested: 9:1, 3:1, 1:3, and 1:9	Total GLA, EPA,and DHA content in diet 8%
11-week-old ovariectomized rats	1:1 ratio of GLA and EPA (diester)	1 g/kg body weight/day. Percentage of GLA/EPA in diet not stated.

reduced PGE2 synthesis and enhanced tibial BMC and collagen cross-link formation but not total bone collagen. PGE2 concentration was positively correlated with total bone collagen and negatively correlated with tibial ash and collagen cross-link formation (61).

Not only is the total amount of LCPUFA in the diet important for optimizing bone formation, but the composition of dietary LCPUFAs also appears to be important. Many studies have focused on determining the optimal ratio of n-6:n-3 fats in the diet for maximizing bone mass.

Table 2. (Extended)

Total dietary fat	Study duration	Outcomes	Reference
8% of diet	42 days	Increased calcium balance and bone calcium content with 3:1 GLA:EPA+DHA	51
10% of diet (100% of total dietary fat was menhaden oil)	40 days	Adverse effect on tibial growth; reduced tibial strength	57
10% of diet (100% of total dietary fat was flaxseed oil)	10 weeks	No effect on serum concentrations of inflammatory cytokines or on bone calcium content or bone mineral density of lumbar spine	59
Diet contained 60 g fat/L	15 days	Increased lumbar spine and whole body bone mineral density with 0.6% dose but not higher doses	60
Diet contained 56.5 g fat/L	15 days	Highest whole body bone mineral density with 0.6 and 0.75% AA	61
Diet contained 57.2 g fat/L	15 days	Increased bone mineral content with either AA/DHA supplementation or PGE2 injection but not combined AA/DHA supplementation and PGE2 injection; reduced bone resorption marker (N-telopeptides of type 1 collagen) and bone PGE2 with AA/DHA supplementation	62
5% of diet (100% of total dietary fat was menhaden oil)	7 months	Higher bone mineral content and collagen cross-link concentration	65
7% of diet	42 days	Increased serum alkaline phosphatase activity (bone formation marker) with higher dietary n-3 LCPUFA content	66
7% of diet	35 days	Increased femur bone mineral density, reduced plasma osteocalcin (bone turnover marker), and reduced PGE2 release from femur	67
11% of diet	12 weeks	Increased femur bone mineral content with 5:1 ratio compared with 10:1 for both high- and low-PUFA diets; no significant effect of amount of PUFA in diet	39
4% of diet	9 weeks	Low dose had no effect on bone mass; high dose resulted in a significant reduction in bone mineral density	42
5.5% of diet	2 months preovx 16 weeks postovx	Reduced bone mineral density loss; prevented ovariectomy-induced increase in RANK-L expression in activated CD4+ T cells	71
3.4% of diet	5 weeks	Bone weight and strength significantly higher in animals fed low-calcium diet with EPA compared with low-calcium diet without EPA	73
11.04% of diet	12 weeks	Reduced serum pyd concentration (bone resorption marker) with low-DHA diet; greater vertebral bone mineral density with high-DHA diet	74
8% of diet	6 weeks	Concentration of DGLA, EPA, and DHA in red blood cell membranes positively correlated with femur calcium content; DHLA concentration negatively correlated with urinary deoxypyridinoline excretion (bone resorption marker)	75
Not stated	14 weeks	No effect on bone mass	76

Watkins *et al.* reported that a ratio of 1.2:1 n-6:n-3 LCPUFAs resulted in a higher rate of bone formation during growth compared with ratios of 23.8:1, 9.8:1, and 2.6:1 (62). Similarly Green *et al.* reported greater BMD in male weanling rats fed a diet containing a 1.4:1 ratio of n-6:n-3 compared with those receiving a 7.1:1 ratio (63). Another study in weanling rats found a 3:1 ratio of GLA:EPA resulted in a lower rate of bone resorption (64) and greater overall calcium balance and bone calcium content, compared with a 1:3 ratio (49).

In young animals, both n-3 and n-6 fats appear to be required for bone growth (57, 62). However, much remains unknown about the effects on bone of individual LCPUFAs within the 2 families. There is some indication that EPA and DHA may have differing bioactivities and/or potencies. In growing male rats, supplementation with tuna oil (high DHA) was more effective than supplementation with fish oil (high EPA) in maximizing bone calcium content (36). High plasma DHA concentration was also associated with lower bone resorption rate in growing piglets (65).

Studies in Ovariectomized Animals. Increased intake of the very long-chain n-3 PUFAs, EPA, and DHA, with (66) or without (35, 67) the n-6 LCPUFA GLA, has been shown to reduce bone resorption (35, 66), inhibit RANK-L expression and inflammatory cytokine synthesis (67), and preserve BMC (35, 66, 67) in ovariectomized rodents. In most cases, the beneficial effect of these interventions on bone mass is attributed to inhibition of PGE2 synthesis and a resultant reduction in the synthesis of inflammatory mediators leading to inhibition of osteoclastogenesis (30). Watkins *et al*. observed that a ratio of 5:1 n-6:n-3 LCPUFAs was more beneficial than a 10:1 ratio in maintaining bone mass after ovariectomy in rats regardless of the total dietary PUFA content (39). However, lumbar spine BMC was preserved in ovariectomized mice fed a diet containing an n-6:n-3 LCPUFA ratio of approximately 1:12 (71), and Kruger *et al*. reported that a 1:3 but not a 3:1 ratio of n-6:n-3 LCPUFAs prevented the ovariectomy-induced decrease in femur BMD and femur calcium content in rats (72). The wide range of n-6:n-3 ratios associated with beneficial effects on bone mass may in part be due to the different animal models used in the various studies. For instance, a 2-month-old, growing ovariectomized rat model was used by Watkins *et al*., whereas Kruger *et al*. used 6-month-old skeletally mature ovariectomized rats. The LCPUFA requirement to optimize bone mass during bone modeling may differ from that required to optimize bone mass during bone remodeling.

As with studies in nonovariectomized, growing animals, there is some evidence that different LCPUFAs within the 2 LCPUFA families may have differing effects on bone in ovariectomized animals. A positive correlation between EPA, DHA, and DGLA concentrations in erythrocyte membranes and femur calcium content has been observed in one study. Erythrocyte membrane DGLA content but not EPA or DHA was also negatively correlated with urinary DPyd excretion, suggesting that DGLA may have an antiresorptive effect (66).

Most studies have utilized supplements containing a mixture of LCPUFAs. However, 2 studies have examined the effects of EPA alone on bone in ovariectomized rats. Ovariectomy-induced bone loss was prevented by supplementation of ovariectomized rats receiving a low-calcium diet (0.01% calcium) with 160 mg of EPA/kg body weight/day. However, no effect of EPA was seen in rats receiving a calcium-adequate diet (73). In a second study, 100 mg EPA/kg body weight/day had no effect on bone mass, whereas 1,000 mg/kg body weight/day increased the rate of bone resorption and had a detrimental effect on lumbar spine and femur BMC in ovariectomized rats receiving a calcium-adequate diet (42). Findings from these 2 studies suggest that EPA may only be beneficial for preserving bone mass after ovariectomy when dietary calcium is limiting. One study has reported that dietary supplementation using a high-DHA oil was more effective than supplementation with a high-EPA fish oil in maintaining bone mass after ovariectomy (74). However, a growing ovariectomized rat model was used in this study. Therefore, whether DHA would be more effective than EPA in maintaining BMC after ovariectomy in skeletally mature animals is unknown.

Mechanisms of Action. *Effect on Calcium Balance.* Findings from both *in vitro* and *in vivo* studies suggest that LCPUFAs may promote intestinal calcium absorption, thereby increasing overall calcium balance. Ca^{2+}ATPase is the enzyme responsible for active calcium absorption in the intestine. The activity of Ca^{2+}ATPase in basolateral membranes from duodenal enterocytes treated with DHA was increased compared with nontreated and EPA-treated membranes (50). The stimulatory effect of DHA was only evident in membranes from which calmodulin was removed, suggesting that DHA may only have a physiologically relevant effect on active calcium transport when dietary calcium intake is low. However, dietary supplementation with either fish oil or evening primrose oil in rats receiving a calcium-adequate diet resulted in increased calcium transport across the basolateral membrane (75) and decreased faecal calcium excretion (68, 76). It is possible that physiological changes brought about by increased membrane content of LCPUFAs led to increased passive, as well as active, calcium transport. The n-6 LCPUFAs may be less effective than n-3 LCPUFAs in promoting calcium absorption because an increase in overall calcium balance has only been observed with fish oil supplementation (68, 76).

There is some evidence that one of the means by which 1,25-dihydroxyvitamin D promotes intestinal calcium absorption involves increasing the concentration of highly unsaturated fatty acids in membrane phospholipids (76). Membrane LCPUFA content is known to affect the structure, fluidity, and polarity of membranes, as well as the relative proportion of membrane-bound proteins (77). Structurally, membranes are composed of "liquid disordered" phospholipid regions interspersed with tightly packed, more orderly "lipid rafts" that consist of sphingolipids and cholesterol. Cholesterol is essential for the formation of lipid rafts (78). Highly unsaturated long-chain fats, such as DHA, have a strong aversion to cholesterol. Incorporation of DHA into a membrane region results in complete expulsion of cholesterol, hence reducing the proportion of lipid rafts in the membrane (77). However, the DHA metabolite 10,17S-docosatriene (protectin D1) has been shown to promote lipid raft clustering in peripheral

blood mononuclear cells (2). Therefore, both the LCPUFA content and the oxidation state of membrane LCPUFAs influences the physiological properties of the membrane.

Altering the dispersion of lipid rafts within membranes modulates the activity of membrane proteins. Membrane proteins can be classified into 3 groups: those that associate with lipid rafts, those that associate with the liquid-disordered regions, and those that can associate with either region depending on their state (78). Lipid rafts are small with few proteins associated with each. In order for ligand-receptor binding to occur, rafts must cluster together, enabling proteins to move laterally within and between rafts (78). Examples of lipid raft-associated proteins include Ca^{2+}-ATPase (79) and components of the NF-κB kinase complex (80). Recently, an oestrogen receptor-like protein similar to ER-α has also been detected within lipid rafts on the plasma membrane of osteoblasts (81). Modulation of the lipid raft content of membranes may be a means by which LCPUFAs alter cellular responses.

Incorporation of unsaturated fats into cellular membranes increases membrane fluidity (82). The greatest increase occurs with the addition of 2 and 3 double bonds, with little change in fluidity occurring with more than 3 double bonds (77). The presence of multiple double bonds allows considerable bending in a fatty acid chain. For instance, oleic acid (18:1) has an average chain length at 41°C of 14.2Å, whereas DHA (22:6) has an average chain length under the same conditions of just 8.2Å (77).

Membrane permeability and the speed of membrane flip-flop (movement of membrane constituents between layers in the membrane bilayer) are increased as the number of double bonds in the fatty acyl chains increases (83). Increased membrane unsaturation may expedite cellular uptake of nutrients and other molecules, particularly by passive transport.

Studies in marine-dwelling bacteria that have the ability to synthesize EPA and DHA under oxygen-limited or anaerobic conditions have shown that EPA or DHA enrichment of membranes supports proton bioenergetics, allowing oxidative respiration and energy transduction (82). Whether this aspect of EPA and DHA activity has any relevance to the mechanism by which they regulate calcium balance or bone metabolism is unknown.

Effect on Osteoblastogenesis and Osteoblast Activity. LCPUFAs and their metabolites regulate transcription of a number of genes *via* the action of peroxisome proliferator activator receptors (PPARs) (84). PPAR-dependent proteins include cytochrome P450, Acyl CoA synthase, fatty acid binding proteins, and various enzymes involved in NADPH production and fatty acid oxidation in peroxisomes and mitochondria (84). LCPUFAs, as well as prostaglandins and various LOX-generated LCPUFA metabolites, are natural PPAR ligands (29, 85, 86). To date, 3 PPARs have been identified: PPAR-α, PPAR-γ, and PPAR-β/δ (85, 87), although at least 2 subforms of PPAR-γ exist (88). All 3 PPARs are expressed by osteoblasts and activation of PPAR-α, PPAR-δ, or PPAR-γ1 in preosteoblasts can promote differentiation into mature osteoblasts (85). In contrast, ligand-mediated activation of PPAR-γ2 promotes differentiation of mesenchymal progenitors into adipocytes rather than osteoblasts (85, 89). Expression in osteoblasts of PPAR-γ1 and synthesis of at least one of its natural ligands, the AA metabolite Δ(12)PGJ(2), is increased in response to mechanical loading (88). DHA and AA are also believed to be PPAR-γ ligands (86), although whether they activate one or both of the PPAR-γ subforms is unknown. Culture of human primary osteoblasts and MG63 cells, a human osteosarcoma cell line, with DHA and AA inhibited cell proliferation as well as apoptosis and resulted in cell cycle withdrawal, possibly as a result of PPAR activation (90). This may indicate a positive effect of the 2 LCPUFAs on osteoblastogenesis because cessation of proliferation and cell cycle withdrawal are characteristic preparative steps for differentiation into the mature osteoblast phenotype (86). In support of this, an increase in alkaline phosphatase activity (a marker of the mature osteoblast phenotype) in MC3T3-E1 osteoblast-like cells after treatment with n-3 fatty acids has been reported (21). In hepatocytes, the LCPUFA metabolites HETE and PGJ(2) promote PPAR-α and PPAR-γ expression (91), raising the possibility that these metabolites may also induce PPAR expression in osteoblast precursors.

Fluid shear stress in osteocytes (92) or exposure of osteoblasts to 17β-estradiol or 1,25-dihydroxyvitamin D_3 (93) results in a rapid increase in intracellular calcium concentration. Phospholipase, which catalyzes the release of AA and DHA from membrane phospholipids (94), is essential for the rise in intracellular Ca^{2+} resulting from 17β-estradiol or 1,25-dihydroxyvitamin D_3 stimulation (93) and may also be involved in increasing intracellular Ca^{2+} in response to fluid shear (92). Prostaglandins and other oxidized LCPUFA derivatives are ionophores (95, 96). Therefore, LCPUFAs may have a role in early-stage activation of osteoblast and osteocyte activity in response to hormonal or mechanical stimuli.

Part of the mechanism by which n-3 LCPUFAs promote osteoblastogenesis appears to be *via* prevention of the formation of products that inhibit osteoblastogenesis. Some n-3 LCPUFAs inhibit 5-LOX activity and nonenzymatic lipid peroxidation. Several members of the leukotriene family, formed by 5-LOX activity, have been shown to inhibit the bone-forming capacity of osteoblasts *in vitro* (97). The isoprostane 8-isoprostaglandin E2, a product of nonenzymatic oxidation of AA, promotes osteoclastogenesis (98) and inhibits osteoblastogenesis in bone but induces osteoblastic differentiation of vascular cells, hence promoting arterial calcification (99). Supplementation with fish oil or EPA has been demonstrated to reduce deposition of calcium in kidneys and the aorta (45), which may be a result of n-3 LCPUFA-mediated inhibition of isoprostane formation.

Feeding a high–n-3 LCPUFA diet to larval European sea bass resulted in accelerated osteoblast differentiation due

Table 3. Summary of Known Bioactivity of Long-Chain Polyunsaturated Fatty Acids and Their Metabolites on Calcium Balance and Bone Metabolism

	Functions in bone
Arachidonic acid	Increases intestinal calcium uptake (75) Promotes osteoblastogenesis possibly by activating peroxisome proliferator activator receptor (PPAR)-γ (86, 90, 115) Increases inducible nitric oxide synthase expression in osteoblasts (101)
Prostaglandin E2	Biphasic effect in low concentrations promotes osteoblastogenesis and in high concentrations promotes bone resorption (62, 63) Increases receptor-activated nuclear-kappa B ligand and decreases osteoprotegerin secretion by osteoblasts (103)
Lekotriene B4	Inhibits osteoblast activity (97, 107, 108) Promotes osteoclastogenesis and osteoclast activity (107–109)
Isoprostanes	Inhibits osteoblastogenesis and promotes osteoclastogenesis in bone (98, 99) Induces osteoblastic differentiation of vascular cells (99)
Lipoxin A4	Inhibits osteoclastic bone resorption (113)
Eicosapentaenoic acid	Increases intestinal calcium uptake (75) Decreases osteoclastogenesis and osteoclast activity (71)
Prostaglandin E3	Similar effects and potency as prostaglandin E2 (104)
Leukotriene B5	Generally less potent than LtB4 in other tissue systems (110); effects in bone unclear
Resolvin E1	Decreases osteoclastogenesis (106)
Resolvin E2	Bioactive effects in bone unknown
Docosahexaenoic acid	Increases intestinal Ca^{2+}-ATPase activity (50) Increases intestinal calcium uptake (75) Promotes osteoblastogenesis possibly by activating PPAR-γ (86, 90, 115) Decreases osteoclastogenesis and osteoclast activity (71)
Protectin D1	Bioactive effects in bone unknown
D-series resolvins	Bioactive effects in bone unknown

to upregulation of BMP-4 and retinoid X receptor-α (RXR-α) (100), suggesting that LCPUFAs may activate the BMP signaling pathway during skeletal development. In osteoblasts, AA promotes mRNA expression of inducible nitric oxide synthase (iNOS), and this effect is prevented by EPA, oleic acid (18:1), and tyrosine kinase inhibitors (e.g., genistein) but not by inhibition of COX, suggesting it is not a result of PGE2 activity (101). Nitric oxide stimulates bone formation and suppresses bone resorption. At high concentrations, however, nitric oxide inhibits both bone formation and resorption (102).

Effect on Osteoclastogenesis and Osteoclast Activity. AA treatment of MC3T3-E1 preosteoblast–like cells resulted in increased secretion of soluble RANK-L and decreased secretion of OPG, probably as a result of PGE2 activity (103). Compared with LA and AA, both DHA and EPA decreased osteoclastogenesis and osteoclast activation in bone marrow cell culture (71). The effects of n-3 LCPUFAs on bone are largely attributed to their inhibitory effect on COX-mediated synthesis of proinflammatory prostaglandins (particularly PGE2). PGE3 derived from EPA has similar potency and bioactivity to PGE2 (104); however, EPA is believed to be a less efficient substrate for COX and/or an inhibitor of COX activity (105). Synthesis of proinflammatory prostaglandins from EPA is therefore less than from AA.

Recent evidence suggests that the effects of EPA on osteoclasts may at least partially be due to activity of the E-series resolvins. Topical application of RvE1 prevented osteoclast-mediated bone loss resulting from periodontitis in rabbits. The mechanism involved inhibition of proinflammatory cytokine and PGE2 secretion and osteoclast formation (106). Whether endogenous resolvins and resolvins of the D-series are capable of a similar inhibitory effect remains to be determined.

Leukotrienes promote bone resorption by stimulating proinflammatory cytokine synthesis (107) and induce osteoclastogenesis by a RANK-L–independent mechanism (108, 109). LtB4 (derived from AA) is generally more potent than LtB5 (110), although the effects of LtB5 on bone cells have yet to be fully investigated. Lipoxins are synthesized by bone marrow cells and have been shown to inhibit some of the actions of leukotrienes (111). In murine models of inflammation, lipoxins are potent endogenous anti-inflammatory mediators (112). Their role in the regulation of bone remodeling is largely unknown. However, topical application of LxA4 in rabbits reduced tissue inflammation and bone loss associated with periodontitis (113), suggesting an inhibitory role on osteoclast-mediated bone resorption.

The docosanoids are a relatively recent discovery and as yet much remains unknown about their potential bioactivity. At least some members of the docosanoid family are bioactive and appear to have a role in the resolution of acute inflammation (28, 113, 114). Whether docosanoids also have a role in regulation of bone resorption or formation remains to be determined. The known effects of LCPUFAs and their metabolites on calcium balance and bone are summarized in Table 3.

An Increased Need for LCPUFAs Postmenopause?

Both lifestyle and life-stage influence LCPUFA metabolism. The activity of Δ-6-desaturase, the rate-limiting enzyme in LCPUFA metabolism, and Δ-5-desaturase reduce with advancing age. LCPUFA desaturation is also inhibited by smoking, diabetes, high sodium intake, corticosteroid use, and biotin deficiency (44, 45). The fatty acid composition of adipose tissue changes with advancing age. A marked increase in the adipose tissue content of AA, DPA, and DHA was evident in women, and to a much lesser extent in men, with increasing age, irrespective of diet (115). Changes in serum phospholipid LCPUFA concentrations are also evident after menopause (116), and recent epidemiological evidence suggests that the fatty acid composition of biological membranes alters after menopause (47). One study reported significantly lower red blood cell membrane content of saturated as well as n-3 and n-6 polyunsaturated fat in postmenopausal, compared with premenopausal, breast cancer patients, with the greatest differences being evident in membrane content of palmitic acid, oleic acid, LA, and DHA (117). DHA concentrations in serum have been found to be higher in women than men (118), and both AA and DHA concentrations were higher in women treated with hormone replacement therapy or the selective oestrogen receptor modulator raloxifene, compared with untreated women (119). Estrogen may increase the synthesis of AA and DHA from their precursors (118, 119). Levels of LA and ALA also decline with age in women and, to a lesser extent, in men (115). As a result, aging and menopause lead to a reduction in the ability of endogenous enzymes to convert ALA and LA into the longer-chain, more highly unsaturated LCPUFAs, such as EPA and DHA. The combination of aging and menopause also results in a change in the physiological fate of dietary LCPUFAs, with apparent greater storage in adipose rather than incorporation into biological membranes. Both decreased synthesis and increased storage of LCPUFAs may result in decreased availability of LCPUFAs for biological processes. Increased intake of preformed, very long-chain PUFAs may be necessary to compensate for the decrease in endogenous LCPUFA synthesis and availability.

Conclusions

Different families of LCPUFA appear to have differing effects on the regulation of bone metabolism. In ovariectomized rodents, increased dietary intake of very long-chain n-3 PUFAs provides some protection against ovariectomy-induced bone loss. However, there is no consensus as to the optimal amount of n-3 LCPUFAs or the optimal ratio of n-3 and n-6 LCPUFAs required to elicit the maximum bone-protective effect after ovariectomy. It is likely that individual LCPUFAs within both the n-3 and the n-6 LCPUFA families have differing bioactivities in bone. More research is required to determine the relative effects of individual LCPUFAs in bone.

Although in animal models of postmenopausal bone loss n-3 LCPUFAs appear to be bone protective, it is unclear if increased n-3 LCPUFA intake can also minimize bone loss in postmenopausal women. There is a need for further randomized controlled intervention studies in postmenopausal women to clarify the effects of dietary intake of the very long-chain n-3 LCPUFAs on bone mineral content and density.

LCPUFAs may influence intestinal calcium absorption, as well as osteoblastogenesis and osteoclastogenesis. Three studies have reported a possible beneficial effect of n-3 LCPUFA supplementation on intestinal calcium absorption or calcium balance in rodents. However, further work is required to determine the mechanism involved and to ascertain whether n-3 LCPUFA supplementation also increases calcium absorption and balance in humans.

LCPUFAs and their metabolites are known to be natural ligands for various PPARs and as a result contribute to the regulation of osteoblastogenesis. The specificity of some AA metabolites for the different PPARs and the resultant effect on osteoblastogenesis is known. However, the effects of EPA and DHA on PPAR activation and osteoblastogenesis are unclear. Both prostanoid and non-prostanoid metabolites of AA stimulate osteoclastogenesis and promote bone resorption by RANK-L–dependent and –independent pathways. Conversely, EPA, DHA, RvE1, and LxA4 have been shown to inhibit osteoclastogenesis. There is some indication that EPA and DHA may modulate RANK-L signalling, although this needs to be confirmed. The mechanism by which RvE1 and LxA4 inhibit osteoclastogenesis remains to be determined.

Members of both the n-3 and the n-6 LCPUFA families, as well as prostanoid and nonprostanoid lipid mediators derived from these LCPUFAs, have been shown to be bioactive in bone *in vitro* and/or in animals. Further research is required to ascertain the relative effects of individual LCPUFAs and their nonprostanoid metabolites on bone and to determine the effects of the amount and composition of dietary LCPUFAs on bone mass in humans.

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