Long chain polyunsaturated fatty acids: selected mechanisms of action on bone

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Abstract :

Evidence presented over the past 20 years have has shown that long chain polyunsaturated fatty acids (LCPUFAs), especially the n-3 fatty acids such as eicospentaenoic acid (EPA) and docosahexaenoic acid (DHA) are beneficial for bone health. Some studies in humans indicate that LCPUFAs can increase bone formation, affect peak bone mass in adolescents and reduce bone loss as measured using bone mineral densitometry. The cellular mechanisms of action of the LCPUFAs, however, are complex and involve modulation of fatty acid metabolites such as prostaglandins, resolvins and protectins, several signalling pathways, cytokines and growth factors. LCPUFAs affect receptor activator of nuclear factor κβ (RANK), a receptor found on the osteoclast which controls osteoclast formation. Lipoxygenase (LOX) generated lipid mediators (resolvins, lipoxins, protectins and docosanoids) have both anti-inflammatory and pro-resolving activities. Both resolvins and lipoxins inhibit inflammation-induced bone resorption. Arachidonic acid significantly upregulates inducible NO synthase (iNOS) mRNA expression in human osteoblast-like cells, thereby possibly enhancing osteoclastic activity. The protective effect of EPA on osteoblastogenesis could be mediated by the biphasic cross-talk between PGE_2 and NO production involving COX-2 and iNOS pathways. Other mediators of osteoblast maturation include PPARα ligands such as linoleic acid and possibly DHA in association with bone morphogenic proteins. Since DHA is a weaker ligand for PPARγ, more uncommitted mesenchymal stem cells are thought to differentiate into osteoblasts rather than adipocytes. This review addresses selected cellular mechanisms that may explain the beneficial effects of the LCPUFAs on bone.

Keywords : long chain polyunsaturated fatty acids ; bone ; osteoclasts ;

osteoblasts ; nitric oxide ; protaglandins, LOX metabolites ; PPAR ligands.

List of abbreviations

#	number
13R-HDHA	13R-hydroxylated DHA
17R-HDHA	17R-HDHA
9,10 DHOA	9,10 dihydroxyoctadecenoic acid
9,10 EOA	9,10 epoxyoctadecenoic acid
9-HODE	9-hydroxyoctadecadienoic acid
AA	Arachidonic acid
ALA	Alpha-linolenic acid
ALP	Alkaline phosphatase
BMP-2	Bone morphogenic protein -2
BMSCs	Bone marrow stromal cells
Cbfa1	Core binding factor α1
CLA	Conjugated linoleic acid
COX	Cyclooxygenase
DGLA	Dihomogammalinolenic acid
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
EFA (s)	Essential fatty acid (s)
EPA	Eicosapentaenoic acid
GLA	Gammalinolenic acid
IGF-1	Insulin-like growth factor 1
IGFBP	Insulin-like growth factor binding protein
IL-1	Interleukin-1
IL-10	Interleukin-10

IL-6	Interleukin -6
LA	Linoleic acid
LCPUFAs	Long chain polyunsaturated fatty acids
LOX	Lipoxygenase
LPS	Lipopolysaccharide
LRP5	Low-density lipoprotein receptor-related protein 5
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage colony stimulating factor
MSC	Mesenchymal stem cell
NF-kappa-β	Nuclear factor beta
NO	Nitrous oxide
NTx	N-telopeptide of Type I collagen
OPG	Osteoprotegerin
OVX	Ovareictomised rats
PGE_2	Prostaglandin E_2
PGE_3	Prostaglandin E_3
PGJ_2	Prostaglandin J_2
PPAR	Peroxisome proliferator activator receptor
PTH	Parathyroid hormone
PUFAs	Polyunsaturated fatty acids
QTL	Quantitative trait locus
RANK	Receptor activator of nuclear factor κβ
RANKL	Receptor activator of nuclear factor κβ ligand
Rt-PCR	Real time PCR....
RvE1	Resolvin E1

RXR	Retinoid X receptors
TGF-β	Transforming growth factor β
TNF-α	Tumour necrosis factor α
TRAP	Tartrate-resistant acid phosphatise
TXA_2	Tromboxane A_2

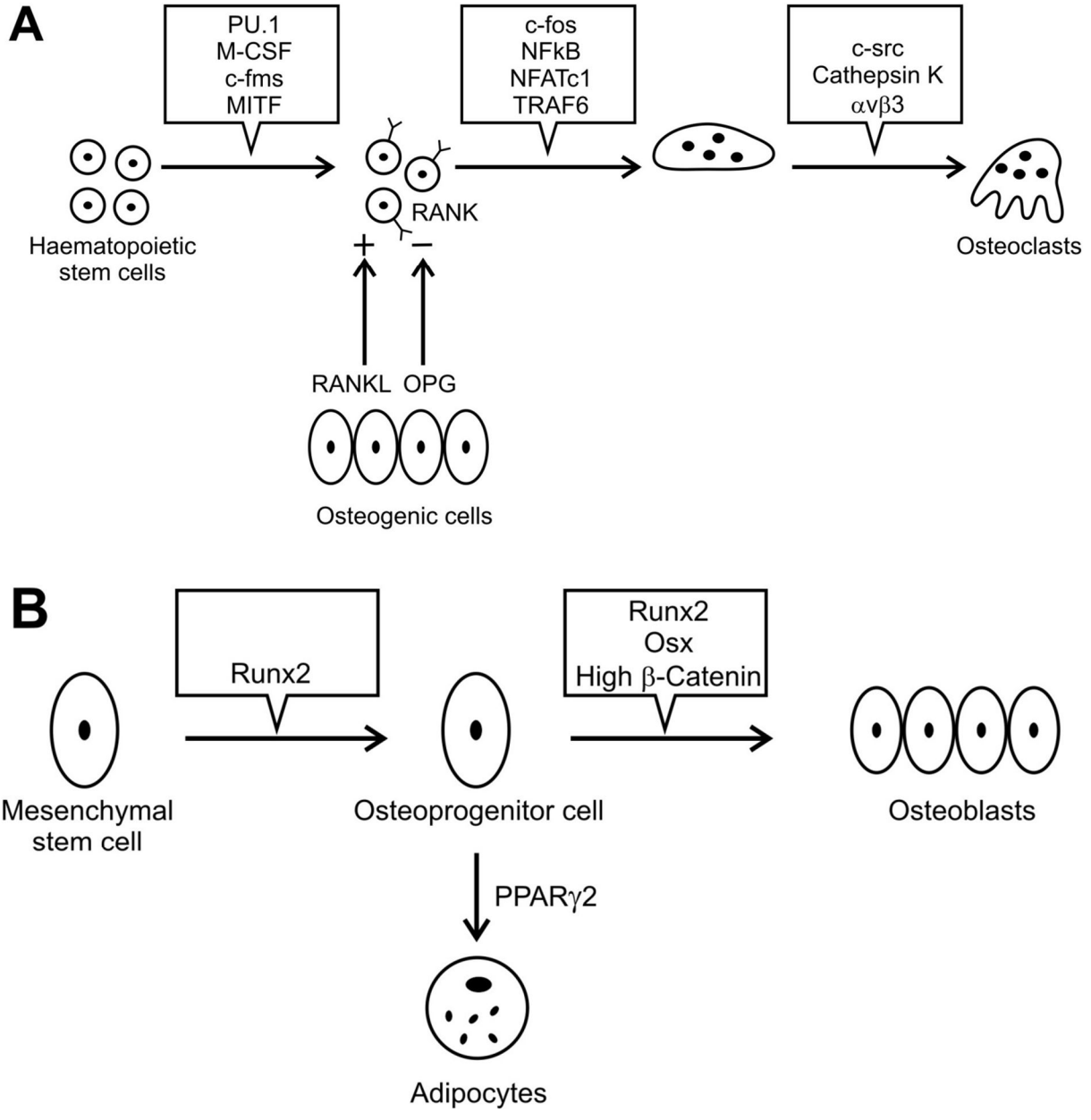
1. Introduction

The mature skeleton is a metabolically active organ that undergoes continuous remodelling by a process that replaces old bone with new bone. I healthy adults, bone resorption and formation are balanced and a constant level of bone mass is maintained. Osteoclasts responsible for bone resorption originate from haematopoetic stem cells know as monocytes, while osteoblasts responsible for bone formation, originate from bone marrow stromal cells. Figure 1 illustrates the differentiation pathways for the osteoblast and the osteoclast. The various factors involved will be described in the review.

Osteoporosis is a systemic bone disease characterised by weak bone material which predisposes the patient to a high risk of fractures [1]. In the older female osteoporosis poses a major health risk due to hip fractures, and also affects the economy due to high cost of treatments and hospitalisation once fractures have occurred. There is accelerated bone loss due to menopause putting these women at risk, but the highest risk for fracture would be in elderly postmenopausal females or in elderly men. Hip fractures, specifically, are debilitating with high morbidity and mortality and like peripheral and vertebral fractures can affect quality of life.

Over the past years the body of evidence to support the notion that dietary long chain polyunsaturated fatty acids (LCPUFAs) with a chain length longer than 18C, are beneficial for bone health [2-4] has been growing. Very early research showed that a deficiency in LCPUFAs could affect bone: In 1931

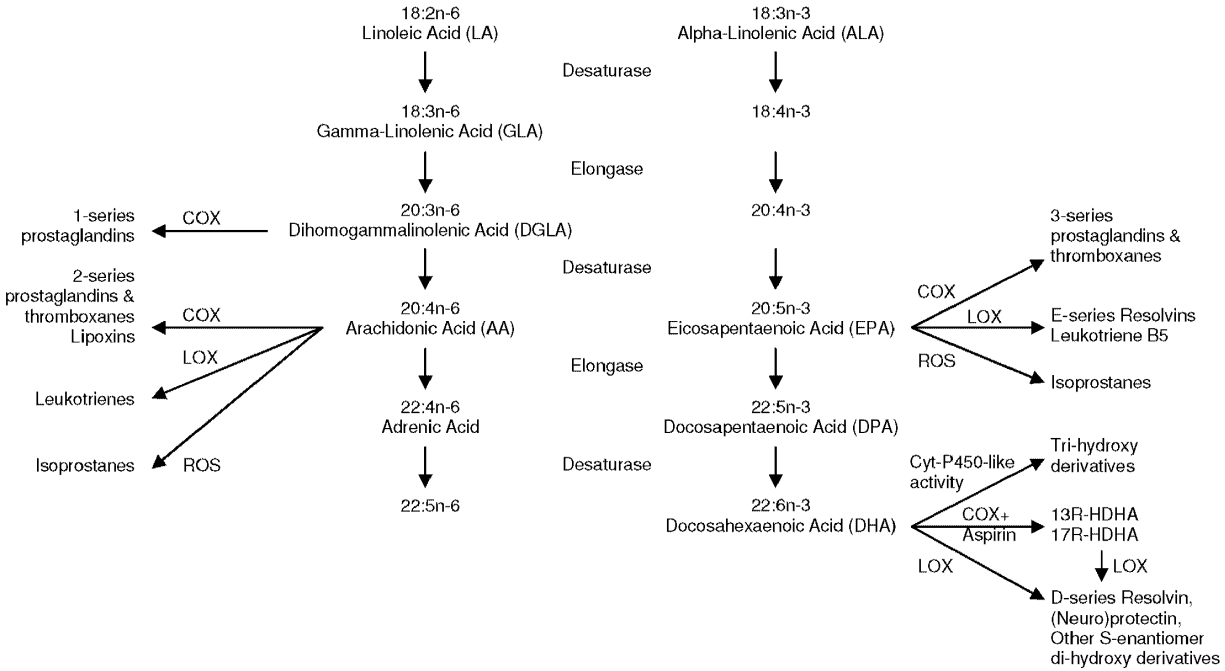
Figure 1: A simplified diagram of the differentiation of (A) osteoclast precursors into osteoclasts and (B) osteoblast precursors into osteoblasts. PU.1, Transcription factor PU.1; M-CSF, macrophage-colony stimulating factor; c-fms, c-fms genes; MITF, microphthalmia-associated transcription factor; c-fos, c-fos gene; NFκβ, nuclear factor κβ; NFATc1, nuclear factor of activated T cells 1; TRAF6, tumor necrosis factor receptor-associated factor-6; c-src, cellular-sarcoma gene; αvβ3, αvβ3-integrin; RANKL, receptor activator of nuclear factor κβ ligand; OPG, osteoprotegerin; Osx, osterix; PPARγ2, peroxisome proliferator activated receptor γ2.



Borland and Jackson [5] reported that essential fatty acid (EFA)-deficient animals were found to develop severe osteoporosis coupled with increased renal and arterial calcification. Studies dating back to 1946 reported that individuals with osteoporosis frequently also had ectopic calcification in other tissues, particularly intervertebral discs, arteries and kidneys [6]. More recently, pathological fractures were reported in newborn rats [7] following dietary EFA deficiency. In a definitive review by Kruger and Horrobin [8] it was suggested that PUFAs of the n-3 series, as well as the n-6 fatty acid gamma linolenic acid (GLA), may prove beneficial when consumed in appropriate amounts. In addition, it has been shown that a reduction of the n-6/n-3 PUFA ratio could result in increased bone strength in animals [9] and in humans [10].

PUFAs are divided into two classes according to their structure. N-3 (ω-3) and n-6 (ω-6) denotes that the third and sixth carbon from the methyl end, respectively, are unsaturated. The longer chain PUFAs can originate from the dietary 18-carbon precursors α-linolenic (ALA,18:3n-3) and linoleic acids (LA,18:2n-6)) respectively and these are considered to be EFAs as they cannot be synthesised by human tissue [11]. Through a process of desaturation and elongation, the longer chain PUFAs are synthesised from these precursors of which the most important ones, with regards to bone health, are arachidonic acid (AA)(20:4n-6), eicosapentaenoic acid (EPA)(20:5n-3) and docosahexaenoic acid (DHA)(22:6n-3)(figure 2).

Figure 2: Metabolism of long-chain polyunsaturated fatty acids. LA and ALA are progressively desaturated and elongated by a shared desaturase/elongase enzyme system to form longer-chain and more highly unsaturated fatty acids. Diagram adapted from Poulsen and Kruger (2007) [152].



Changes in dietary PUFAs are reflected in the composition of various tissues, including bone cells such as the osteoblasts [12, 13]. In line with this finding, the PUFA composition of cell membranes is dependent on the dietary intake to a great extent. It has been shown, for instance, that when humans ingest fish products, the n-3 PUFAs EPA and DHA from the diet partially replace the n-6 PUFAs, especially AA, in cell membranes [14, 15].

ALA is essential in the human diet because it is the substrate for the synthesis of longer-chain, more unsaturated n-3 fatty acids, principally EPA and DHA. The review of Burdge and Calder [16] reported that, despite differences in study design, the physical form in which ALA was presented, and duration of studies, EPA increased in both plasma and cell lipids when ALA intake increased. The extent of conversion to DHA, however, is insufficient to increase the concentration of this PUFA in the tissues meaningfully [16]. Although the affinity of Δ^6-desaturase for ALA is greater than for LA, the relative excess of LA in the average Western diet, and therefore in cellular pools, results in greater conversion of n-6 PUFA [16]. Interestingly, the conversion of ALA to EPA and DHA is greater in women compared to men, possibly due to a regulatory effect of oestrogen [17].

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 as well as non-enzymatic oxidation. There are two 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 [18-20]. 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 tri-hydroxylated derivatives of DHA and include the docosatrienes, protectins (also known as neuroprotectins) and the D-series resolvins [21]. COX converts dihomogammalinolenic acid (DGLA; 20:3n-6), AA and EPA into prostaglandins of the 1-, 2- and 3-series respectively. COX also catalyses the conversion of AA to thromboxane A_2 (TXA_2) [22] and in conjunction with aspirin, the mono-hydroxylation of DHA to form 13R- and 17R-hydroxylated DHA (13R- and 17R-HDHA) [23](figure 2). To date, two distinct *cox* genes have been identified encoding two isoforms of COX known as COX-1 and COX-2 [23]. COX-1 is constitutively expressed in most tissues whereas COX-2 is the inducible form of the enzyme. COX-1 and 2 have greater specificity for AA than EPA therefore preferentially synthesise 2-series rather than 3-series prostaglandins [24].

Several of the LCPUFAs have been shown to affect bone cells, i.e. osteoclasts and osteoblasts, via varying cellular signalling pathways or growth factors, thereby affecting bone formation, resorption and bone density in animals or humans. Salari et al. [11] summarised several studies as part of a meta-analysis of available data. They proposed that dietary intake of precursors for the n-6 and n-3 pathways is essential and modulates membrane composition. Changing the balance of PUFAs present in the membranes towards the n-3 rather than n-6 shifts the ratio of n-6/n-3 in the

membranes and affects production of several cytokines which could affect bone resorption and formation. LCPUFAs have been shown to affect cellular proteins and receptor activator of nuclear factor κβ (RANK), a receptor found on the osteoclast which controls osteoclastogenesis. This will be discussed in detail below [25-27].

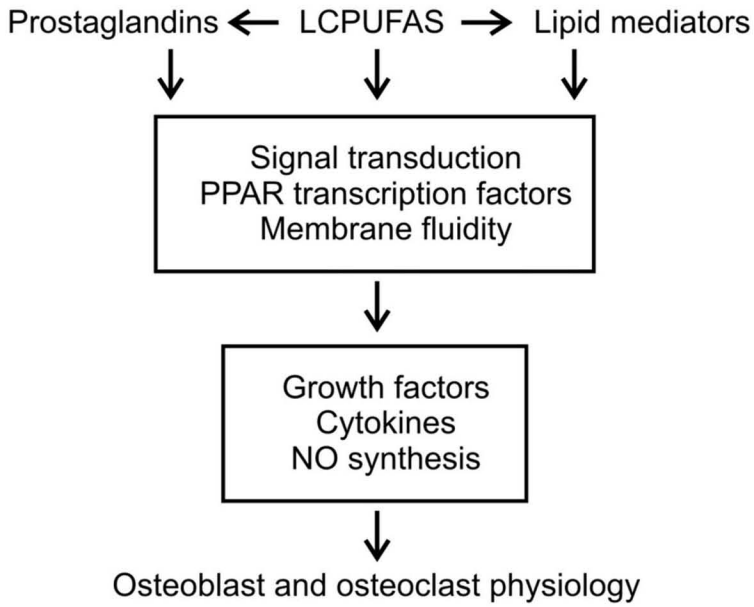
A large number of studies have been done on animals which show that in growing animals PUFAs affect bone mineral content, bone mass, femoral thickness and bone area, seemingly affecting bone formation and growth. N-3 PUFAs also increase alkaline phosphatase (ALP) activity in growing male rats as well as insulin-like growth factor I (IGF-1) and insulin-like growth factor binding protein (IGFBP) levels ([28-30]. Both n-6 and n-3 PUFAs seem to be required for growth. Lau et al. [31] recently made use of the *fat-1* mouse model that endogenously synthesizes n-3 PUFA from n-6 PUFA to determine if outcomes of bone health were correlated with n-3 PUFA in femurs. Young rats of both genders, three weeks of age, were fed a diet containing 10% safflower oil, high in LA, for a period of 12 weeks. Thereafter, femur bone mineral content and density and biomechanical strength properties were determined. Results from this study showed that *fat-1* mice have higher levels of n-3 PUFA and a lower n-6/n-3 PUFA ratio in femur tissue relative to wild-type mice. A reduction in the n-6/n-3 PUFA ratio and an increase in n-3 PUFAs from EPA and DHA may be associated with greater bone strength in this model [31]. Whilst the majority of the work has been done in rats and mice, a study in chickens found no effect by varying ratios of n-3 to n-6

PUFAs on tibial strength or density although a slight effect was found on tibial cortical thickness [32].

During the past 10 years several authors have reviewed various studies investigating the effects of PUFAs on bone health in animals [11, 24, 29, 33-37]. However, few studies in humans have been done despite the strong evidence gleaned from work on animal models. Some human studies indicate that PUFAs can increase bone formation, affect peak bone mass in adolescents [38] and reduce bone loss as measured using bone mineral densitometry [39]. In contrast, some of the studies in humans did not find any effect on bone markers [40] while some showed reduction in bone loss by measuring deoxypyridinolines and N-telopeptide of Type I collagen in the urine [8, 38, 39, 41]. Two studies found a positive correlation between monounsaturated fat in the diet and bone density [42, 43]. This is supported by Martinez-Ramirez et al. [44] who demonstrated that a higher ratio of monounsaturated fat in the diet was associated with a reduced risk of fracture in the elderly. The same study also showed an increased risk of fracture with a high intake of omega 6 fatty acids this population. In addition, Corwin et al. [45] demonstrated that saturated fat in the diet is inversely related to bone density. These studies and others have been extensively reviewed by Salari et al. [11].

The mechanism of action of the n-3 PUFAs is complex and involves several signalling pathways, cytokines and growth factors. These will be reviewed in detail below (figure 3).

Figure 3: A summary of the selected mechanism of action of long-chain polyunsaturated fatty acids on bone cells to be covered in the current report. Membrane fluidity and the role of cytokines have been reviewed in Refs. [8, 11,120,152].



1.1 Effects of dietary polyunsaturated fatty acids on prostaglandin secretion and bone status

Prostaglandins are metabolised from PUFAs (Figure 2) and are considered fast-acting local hormones, often displaying biphasic properties. PGE_2 is derived from the n-6 PUFA AA and it is the major prostaglandin in bone. It has been shown to be a potent modulator of bone remodeling, affecting both bone resorption [46] and formation [47, 48]. Excessive production of PGE_2 may affect bone modeling adversely, whereas a lower level of PGE_2 is believed to stimulate bone formation in animals fed diets containing moderate levels of n-6 PUFAs [49]. Varying the ratio of the precursor fatty acids in the diet is an effective way to modify prostaglandin production in the body. Since n-3 and n-6 fatty acids serve as substrates for the same enzymes along the conversion pathways but are metabolised at different rates [8] lowering the dietary (n-6)/(n-3) PUFA ratio can reduce PGE_2 production [2, 9, 30, 50]. The n-3 PUFAs are precursors of PGE_3 which is equipotent to PGE_2 in bone resorption [51]. However, conversion to prostaglandins from n-3 PUFAs is less effective than from n-6 PUFAs, resulting in lower PGE_3 levels [51, 52]. PGE_2 production can also be reduced by provision of the n-6 PUFA GLA [49]. In addition to reducing synthesis of PGE_2, dietary GLA can enhance production of PGE_1, which has anti-inflammatory effects that could also benefit bone [49, 53]. Combining GLA and EPA in the diet significantly increased serum levels of EPA, but did not increase serum levels of AA, suggesting that a GLA/EPA supplement combination may be implemented to reduce the harmful synthesis of proinflammatory AA metabolites in the body [54]. Investigation of prostaglandin synthesis by bone tissue showed that providing n-3 PUFA in the

diet will reduce ex vivo PGE_2 synthesis in rat femurs, while also reducing circulating pyridinoline levels in the blood. These observations indicate an effect of n-3 PUFAs on bone resorption by reducing production of pro-inflammatory PG's [37].

Several nutritional fatty acid studies on growing piglets have been conducted since 2000 [50, 55-57]. These studies collectively show that modulation of the dietary ratio of n-6 versus n-3 PUFA may change the bone fatty acid profile and elevate ex vivo PGE_2 release from bone, whilst higher DHA in the diet may lower bone resorption as assessed by the measurement of urinary N-telopeptide [55, 58]. When comparing the effect of dietary PUFAs to that of a low dose of exogenous PGE_2, the latter enhances osteoblast activity and reduces urinary calcium excretion. This observation, together with the previous data that additional dietary AA and DHA reduce bone resorption, leads to the conclusion that both PUFAs and low dose PGE_2 could enhance mineral content in the growing piglet model, but through distinct separate mechanisms [55].

Watkins et al. [59] have shown that feeding chicks menhaden oil (high in n-3 PUFAs) resulted in a higher serum ALP activity and an increase in the bone formation rate compared to that in chicks given soy-bean oil (high in n-6 PUFAs). The effect of PUFAs might be exerted via modulation and reduction of PGE_2 synthesis, reducing inflammation and affecting ALP activity [2, 59]. Watkins et al. [24] thus suggested that by lowering the dietary (n-6)/(n-3)

PUFA ratio, PGE_2 production could be reduced and bone formation therefore enhanced.

The mechanisms by which PGE_2 exerts its effects on bone are still under investigation. Recently, *in vivo* animal studies examining the effects of varying doses, schedules and routes of administration of PGE_2 on bone have been reported. For example, Tian et al. [60] showed that continuous PGE_2 infusion to rats led to bone loss by stimulating bone resorption to a higher degree than formation and shortening the formation period. In contrast, intermittent PGE_2 administration resulted in bone gain due to stimulated bone formation and shortened resorption periods [60]. These results were confirmed in male C57BL/6 mice, where decreased trabecular bone volume was reported after 14 days treatment with a high dose of PGE_2 (6 mg/kg/day), suggesting increased bone resorption. Furthermore, brief exposure to high dose or prolonged exposure to lower dose of PGE_2 resulted in a significant increase in bone formation rate [61].

However, disparate results on bone formation, osteoclastogenesis and bone resorption have been reported in *in vitro* systems, depending on the model and concentration of PGE_2 used. PGE_2 has been shown to stimulate osteoclastogenesis in murine bone marrow cultures [62] and murine spleen cell cultures treated with receptor activator of nuclear factor κβ ligand (RANKL) and macrophage colony stimulating factor (M-CSF) which may be attributed to stimulation of adenylate cyclase mediated by the EP_2 and EP_4 receptors [63]. Time course experiments showed biphasic effects of PGE_2 on osteoclastogenesis with an initial inhibitory effect and a later stimulatory effect

in a model of murine spleen cell cultures. In this system, PGE_2 also increased osteoclast size and resorptive pit formation and decreased osteoclast apoptosis [64, 65]. Recently Tsutsumi et al. [65] showed that fibroblasts, which are considered a predominant source of RANKL, cannot express RANKL after stimulation with PGE_2 in the absence of the EP4 receptor in a conditional knockout murine model. These results confirm that PGE_2 signaling in fibroblasts is mediated through the EP4 receptor resulting in upregulation of RANKL and stimulation of osteoclastogenesis.

Offering a novel mechanism of PGE_2 action, Liu et al. [66] indicated that PGE_2 and IL-6-induced osteoclastogenesis occurs via inhibition of osteoprotegerin (OPG) secretion and stimulation of RANKL production by osteoblasts as well as upregulation of RANK expression in osteoclasts. IL-6 and PGE_2 induce each other's production as well. Furthermore, PGE_2 effects on osteoclastogenesis in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells have been reported by Kaneko et al. [67]. PGE_2 dose-dependently increased osteoclastogenesis by affecting the hematopoetic cell lineage. Addition of NS398 blocked the response to PGE_2 indicating COX-2 activity. In contrast, a study by Take et al. [68] showed that the conditioned media of human CD14+ monocytes, pretreated with PGE_2, inhibited RANKL-induced osteoclast formation, probably through the production of an inhibitory factor for osteoclastogenesis (of osteoclast precursors).

Regarding osteoblasts, Minamizaki et al. [69] recently reported PGE_2-stimulated proliferation and differentiation of osteoblastic precursor cells in the

rat calvarial cell model. This anabolic effect of PGE_2 on bone formation may be mediated through multiple EP_2 and EP_4-MAPK signalling pathways.. PGE_2 has also been shown to promote survival of rat bone marrow stromal cells by preventing their apoptosis via binding the EP_4 receptor, activating sphingosine kinase and inhibiting caspase activity [70].

When mouse osteoblast and bone marrow co-cultures were treated with LPS, PGE_2 synthesis and osteoclast formation followed, both effects could be blocked by the COX-2 inhibitor NS398. In addition, LPS induced stimulation of RANKL mRNA expression and inhibition of OPG mRNA expression in the same system, suggesting that down-regulation of OPG expression by PGE_2 is involved in LPS-induced osteoclastogenesis [71].

1.2 Effects of dietary polyunsaturated fatty acids and their prostaglandin metabolites on growth factors

Transforming growth factor-β1 (TGF-β) has been believed to regulate differentiation, enhance proliferation and induce anabolic activity in osteoblasts [72]. Osteoblasts secreted TGF-β dose-dependently when stimulated with varying concentrations (10^{-7} to 10^{-3} M) of PGE_2, a metabolite of AA. The same study also showed that TGF-β1 receptor expression in osteoblasts increases after 10 days of exposure to PGE_2 at low concentrations (10^{-5} and 10^{-7} M) thereby permitting higher binding of TGF-β to its receptors, which means less activated TGF-β is free in the extracellular media. Higher concentrations of PGE_2 (10^{-3} M) produces the opposite effect

[44]. These authors argued that lower PGE_2 concentrations stimulate TGF-β RI gene expression that concomitantly results in increased binding of free TGF-β to these receptors, resulting in increased osteoblastic activity and more bone deposition. Using *in vitro* and *in vivo* mouse models, Tang et al. [73] demonstrated recently that active TGF-β1 released from bone matrix during bone resorption induces migration of osteogenic bone marrow stromal cells (BMSCs) to the resorption sites. The BMSCs then undergo osteoblastic differentiation in response to signals provided by the microenvironment of the resorptive sites. TGF- β interacts with three distinct cell surface receptors (TβRIs, TβRII and TβRIII). TβRIII lacks a distinct intracellular signaling motif and may control the stability, ligand binding capacity and signal generation of TβRI and TβRII [74]. In primary fetal rat osteoblasts it has been shown that high levels of PGE_2 may increase TβRIII expression and in this way delay TGF- β-dependent activation of osteoblasts during the initial stabilization phase before cell replication and bone repair [75].

Insulin-like growth factors (IGFs), especially IGF-I, are major bone-derived growth factors and are believed to function as both systemic and local growth factors for bone tissue. Once secreted and deposited in bone matrix, IGFs are released during osteoclastic bone resorptive activity, acting in an autocrine or paracrine fashion to stimulate new bone cell formation and matrix production [76]. IGF-I acts as a regulator of bone cell function as it stimulates the proliferation of pre-osteoblasts, thereby increasing the number of cells capable of producing bone matrix. In addition, IGF-I increases collagen expression while decreasing collagen degradation, causing an anabolic effect

in bone tissue [77, 78]. In osteoblasts, hormones such as growth hormone, parathyroid hormone and oestrogen modulate IGF-I expression [78]. In addition to IGF-I, osteoblasts also synthesise extracellular high affinity IGF-binding proteins (IGFBPs), which modify the interaction of IGF-I with its receptors by prolonging IGF stability and by influencing ligand-receptor interaction [79, 80]. IGF-1 is deemed necessary for maintaining the normal interaction between the osteoblast and osteoclast to support osteoclastogenesis through regulating RANKL and RANK expression [81].

Dietary PUFAs may up-regulate or down-regulate IGF-I production in bone via their ability to modulate local concentrations of the AA metabolite PGE_2 [30, 48, 78]. PGE_2 also promotes the expression of various IGF-binding proteins [77, 80, 82] suggesting that PGE_2 could keep IGF available for stimulation of osteoblasts at a later phase of bone remodeling [77]. McCarthy et al. [48] suggested that the ability of PGE_2 to enhance osteoblastic IGF-I synthesis could explain its anabolic potential, and furthermore suggests a role for PGE_2 in coupled bone remodeling. The anabolic effects of PGE_2 may occur through stimulation of endogenous IGF-I production by osteoblasts [83] or by increased bone cell responsiveness to IGF-I [84].

Li et al. [30] showed that feeding a fish oil-enriched diet to rapidly growing male rats increases the serum concentration of IGFBP-3, an important modulator of IGF-I and overall bone growth and development. Though little data on the in vitro effect of PUFAs on IGF-1 are available, IGF-1 may be involved in the observed effects of PUFAs on osteoclastogenesis. PUFAs

such as AA, EPA and DHA inhibit both osteoblast and Caco-2 adenocarcinoma cell proliferation [26, 85]. EPA and DHA both reduced IGF-II and increased IGFBP-6 in Caco-2 cells resulting in less free IGF-II thereby inhibiting cell proliferation [85]. It can thus be speculated that the latter may also be the mechanism involved whereby EPA and DHA suppress osteoblast proliferation.

2. Effects of polyunsaturated fatty acids on bone cell growth and differentiation

2.1 Effects of polyunsaturated fatty acids on early osteoblastic differentiation

Atkinson et al. [13] demonstrated that feeding post-weanling male Fisher rats DHA had substantial bone marrow enhancing activity, resulting in a two-fold increase in bone marrow cell number over n-6 PUFA fed animals. Bone marrow contains various precursor cells including mesenchymal stem cells that are able to differentiate into several cell types including osteoblasts and adipocytes [86, 87]. One could therefore speculate that higher numbers of bone marrow cells could increase the potential for osteoblastogenesis, provided the required transcription factors are expressed. Commitment of a mesenchymal stem cell (MSC) to the osteoblastic lineage is regulated by specific transcription factors of which core binding factor α-1 (Cbfa1), also known as Runx2, has been identified as the earliest and most specific marker of osteogenesis [88, 89], and also as the master switch in osteoblast differentiation [90]. However, other signals in pre-osteoblasts such as IGF-1 and PTH (stimulatory) and TNFα (inhibitory) mediate Cbfa1 translocation into

the nucleus resulting in altered osteoblast differentiation. Serum TNFα is reduced by DHA [91] and interestingly, in middle-aged rats, n-3 LCPUFA enhanced PTH and IGF-1 [37]; all of which would enhance Cbfa1 translocation to the nucleus and thereby stimulate OB differentiation and expression of proteins such as ALP (early marker), osteocalcin, a late marker.

In 2003 Watkins et al. [33] reported regulatory effects of PUFAs on Cbfa1 expression in fetal murine calvarial osteoblasts: AA, EPA and LA stimulated Cbfa1 expression but conjugated linoleic acid (CLA) decreased protein levels for Cbfa1 after 14 days of treatment. It was speculated that the observed stimulatory effects of AA and LA in this experimental model could be mediated by elevated PGE_2 production. The possible involvement of PGE_2 in Cbfa1 expression is supported by findings of Zhang et al. [92] who reported significant inhibition of Cbfa1 expression in a COX-2 knockout mouse model, which was reversed by the addition of PGE_2. In addition, it has been shown that PGE_2 induces expression of Cbfa1 as well as bone morphogenetic protein-2 (BMP-2) through activation of the EP_4 prostaglandin receptor [93]. Bone-morphogenetic proteins (BMPs) are members of the transforming growth factor-β superfamily and are considered important regulators of the differentiation of uncommitted mesenchymal cells into osteoblasts during both embryonic development and bone repair [94, 95]. Zhang et al. [92] speculated that PGE_2 might induce BMPs and/or cooperate with BMPs to increase Cbfa1 and osterix, two essential transcription factors required for bone formation. However, whether dietary PUFAs could affect BMP-2 expression via modulation of PGE_2 synthesis, is as yet not known. Further

indirect evidence stems from studies where 15d-PGJ2, an alternative product of AA metabolism, reduces expression of Runx2 mRNA via PPARγ signalling [96].

Other signaling in osteogenic precursors from MSCs include Wnt/LRP5 and β-catenin [90]. Beta-catenin is an important downstream mediator in Wnt/LRP5 signaling in bone leading to Cbfa1-mediated osteoblast differentiation. While many signals are convergent in osteoblasts, reduction in β-catenin leads to osteopenia [97]. High DHA reduces β-catenin in carcinoma cells suggesting a possible explanation for the adverse effects of very high n-3 LCPUFA in early development.

2.2 Effects of polyunsaturated fatty acids on osteoclastogenesis

Osteoclastogenesis is controlled by three proteins. Two are associated with the osteoblasts namely RANKL which is expressed on the osteoblast cell membrane, and OPG which is secreted by the osteoblast. RANK is a membrane-bound receptor found on osteoclast precursors and it is activated by binding to RANKL, leading to osteoclastogenesis. OPG acts as decoy receptor secreted by the osteoblasts and can prevent osteoclastogenesis by binding to RANKL in high concentration [98].

Recently Poulsen et al. [99] showed that COX products from AA and GLA induce RANKL in mouse MC3T3-E1/4 cells whilst EPA and DHA did not affect RANKL. Therefore AA and GLA may induce osteoclastogenesis. Similarly, Coetzee et al. [26, 100], showed that AA inhibited the secretion of OPG by

osteoblasts and this inhibition was attenuated by a COX inhibitor indomethacin, pointing towards the involvement of prostaglandin synthesis from AA. AA stimulated RANKL secretion in this system, thus decreasing the OPG/ RANKL ratio. DHA also suppressed OPG secretion but had no effect on RANKL secretion. These results suggest that AA upregulates osteoclastogenesis by affecting the OPG/ RANKL ratio while DHA does not seem to have a significant effect on either OPG or RANKL. In mice prone to autoimmune disease (MRL/Mpj-*Fas*^{lpr}), RT-PCR of lymph nodes revealed significantly reduced RANKL mRNA expression and enhanced OPG mRNA expression after feeding of fish oil. This supports the notion that the bone protective effect of n-3 PUFA may be due to altering the RANKL/OPG ratio [101]. Bone resorption is also increased due to increased expression of NF-kappa-β activity inducing osteoclastogenesis. Rahman et al. [102] reported that n-3 PUFAs reduce NF-kappa-β expression and modulate RANKL signalling whilst n-6 PUFAs such as AA increase NF-kappa β expression leading to increased osteoclastogenesis.

Exposure of cell cultures from primary murine bone marrow cells and RAW 264.7 cells to EPA or DHA or both, and examining TRAP (tartrate-resistant acid phosphatase) activity which is considered to be a marker of osteoclast maturation, showed a significant decrease in osteoclast maturation compared with the n-6 PUFAs LA and AA [27, 102]. The observed inhibition correlated with inhibition of several osteoclast-specific genes such as TRAP, cathepsin K, c-FOS as well as TNF-α. Pretreatment of these cells with DHA caused reduced activation of NF-kappa-β and p38MAPK compared to EPA. DHA

therefore may be much more effective than EPA in alleviating RANKL induced proinflammatory cytokine production, though EPA did have a small effect. The observed effect of EPA may be due to the activity of the E-series resolvins, as topical application of RvE1 prevented osteoclast-mediated bone loss [103].

In contrast to the work reporting effects of LCPUFAs on bone cells, Cornish et al. [104] recently reported an effect of saturated fatty acids on osteoclastogenesis. They studied several short and medium chain fatty acids and stearic (C18:0) and palmitic acid (C16:0) exhibited a significant inhibitory effect on osteoclastogenesis. None of the saturated fatty acids affected RANKL or OPG expression consistently.

2.3 Effects of polyunsaturated fatty acids and metabolites on cytokine secretion and expression

Some workers suggest that the immune system is linked to bone loss. IL-6, IL-1 and tumour necrosis factor alpha (TNF-α) affect the formation and activity of the bone-resorbing osteoclasts and therefore play an important role together with other cellular proteins to control bone resorption. Several cytokines including IL-6 are generated during menopause and could be responsible for a large percentage of the observed increase in bone resorption [105]. Modification of the proinflammatory cytokine profile by PUFAs could thus have a positive effect on bone growth and development [24, 33, 36, 105-109].

A large body of literature now points to the fact that the omega-6 AA increases adverse cytokine production while various omega-3 fatty acids can reverse this trend in bone cells. In vitro exposure of osteoblasts to various PUFAs showed that AA (25 to 100 μM) stimulated expression of IL-1α, IL-1β, TNF-α and M-CSF. EPA and oleic acid (25 to100 μM) on the other hand, had no stimulatory effects, but caused a significant inhibition of AA-induced cytokine mRNA expression [110]. In this system, results from inhibitor studies suggested that a protein kinase C-dependent mechanism could account for the effects of AA on cytokine production. Bordin et al. [111] also showed that AA increases IL-6 expression this was shown to occur via protein kinase Cα activation.

This trend is corroborated by the results of Skuladottir et al. [112] that show that n-3 PUFAs decrease TNF-α secretion by RAW 264.7 macrophages which are precursors for osteoclasts in culture. Furthermore, the study of Bhattacharya et al. [113] showed that when ageing C57BL/6 mice were fed fish oil for 6 months it resulted in higher bone density as well as a decreased activity of pro-inflammatory cytokines IL-6 and TNF-α in concanavalin-stimulated splenocytes. In an elegant study Cohen et al. [114] reported data that IL-10(anti-inflammatory) knock-out mice fed flax seed oil, providing ALA, had significantly lower serum levels of TNF-α and higher bone density compared to IL-10 knock-out mice fed corn oil that is rich in omega-6 fatty acids. The difference in bone density was possibly partly due to the reduction in TNF-α by feeding of ALA. Downregulation of resorptive cytokines such as TNF-α and IL-6 by n-3 PUFA in OVX mice was also reported by other

research groups [115, 116]. Thus dietary modulation by increasing n-3 PUFA intake could affect the generation of IL-1, IL-6 and TNF-α which in turn could modulate bone health. [11, 34, 117].

PGE_2, an AA metabolite, has also been shown to stimulate IL-6 production [15]. There is *in vitro* evidence of cross-talk between the COX-2/PGE_2 and IL-6 systems [118] in murine osteoblast-osteoclast co-cultures that results in increased osteoclastogenesis via effects on the RANK/RANKL/OPG system. Furthermore, it has been shown that co-treatment with AA and EPA both at 10μM, decreased the PGE_2 synthesis observed with AA alone by more than 50% in NIH3T3 fibroblasts [119]. This study also reported that although PGE_2 (derived from AA) and PGE_3 (derived from EPA) both stimulated IL-6 secretion in the pre-osteoclastic RAW 264.7 macrophage cell line, the effect of PGE_3 was significantly lower than PGE_2 in inducing IL-6 secretion in these cells.

3. Further potential PUFA action mechanisms

3.1 Effects of lipid mediators originating from n-3 PUFA

Lipoxygenase (LOX) generated lipid mediators (resolvins, lipoxins, protectins and docosanoids) have both anti-inflammatory and pro-resolving activities [120, 121]. Both resolvins and lipoxins inhibit inflammation-induced bone resorption and may therefore provide a mechanism by which the n-3 PUFAs protect against bone loss. Hasturk et al. [122] reported that RvE1 protects from local inflammation and osteoclast-mediated bone destruction in

periodontitis. Herrera et al. [123] used mouse bone marrow cultures to investigate the effect of RvE1 on osteoclast differentiation and cell survival. RvE1 decreased osteoclast growth and pit formation as well as osteoclast differentiation. Further possible involvement of these lipid mediators in bone metabolism was suggested by a study which showed that following ovariectomy (OVX) of female rats, AA and AA-derived mediators were higher in bone marrow from the OVX rats compared to sham rats. DHA and EPA supplementation of OVX rats increased the percentage of EPA and DHA in the bone marrow and increased the proportion of LOX mediators generated from DHA and EPA respectively. These changes in mediator profile due to OVX and subsequent improvement of anti-inflammatory mediator levels may have consequences for bone metabolism [25].

3.2 Effects of polyunsaturated fatty acids and their metabolites on NO secretion and NO synthase

NO is synthesized from L-arginine and O_2 by NO synthase (NOS) in almost all mammalian cells. Like PGE_2, NO is a cell-signalling molecule with diverse roles in a variety of biological processes [124]. NO is released by osteoblasts and directly regulates osteoclastic activity. NO has also been shown to modulate the effects of proinflammatory cytokines at the skeletal level and is essential for the stimulatory activity of IL-1 on bone resorption both *in vitro* and *in vivo* [125]. NO has been shown to decrease the RANKL/OPG equilibrium, suggesting an inhibitory effect on osteoclastogenesis and bone resorption in ST-2 murine stromal cells [126]. It has been postulated that the

effects of NO on osteoblasts and osteoclasts may depend on its local concentration: at low concentrations (associated with endothelial NO (eNO) or constitutive NO (cNO) synthase activity), NO promotes the proliferation of osteoblasts and prevents osteoporosis, whereas high concentrations (associated with the induction of inducible synthase (iNOS)) may enhance bone resorption by promoting osteoclast formation and activity and suppressing osteoblast proliferation [127]. PUFAs also play a role in this context: It has been reported that AA, unlike EPA and oleic acid, significantly upregulated inducible NO synthase (iNOS) mRNA expression in human osteoblast-like cells, thereby possibly enhancing osteoclastic activity [125]. It has been speculated that the protective effect of EPA on osteoblastogenesis could be mediated by the biphasic cross-talk between PGE_2 and NO production involving COX-2 and iNOS pathways [124].

3.3 Fatty acids and PPARs in bone

Amongst the important mechanisms of PUFA action are their effects on gene transcription [128, 129]. These can be mediated by fatty acid binding to the peroxisome proliferator activator receptor (PPAR) transcription factors that have important effects on bone physiology.

The PPARs were initially described as targets of xenobiotic compounds that promoted proliferation of peroxisomes (cytoplasmic organelles that are active in fatty acid catabolism) in the liver, [130] and are ligand-activated nuclear receptors that belong to the steroid hormone receptor superfamily [131]. PPARs bind to DNA as an obligate heterodimer together with one of the

retinoid X receptors (RXR) [132, 133]. The PPARs exist as the PPAR isoforms α, β/δ and γ. All three are expressed in bone tissue but specifically PPARγ is of importance for adipose and bone tissue function [134, 135].

3.3.1 The role of PPARs in bone cells

PPARγ has been reported to regulate IGF-1 function [136], of major importance in bone, as discussed previously in this review. In addition, the inhibitory role of PPARγ on maturation of various osteoblastic cell lines concomitant with adipocytic differention has been well established [137-139]. Lin et al. [140] have presented evidence that PPARγ inhibits osteogenesis via the down-regulation of COX-2 expression. However, the phenomenon of decreased osteoblast function concomitant with bone adipogenesis seems to be dose dependent : in the MC3T3-E1 pre-osteoblast cell line [141], lower levels of PPARγ activators *induced* alkaline phosphatase activity, matrix calcification and the expression of osteoblast genes, but higher levels of the specific PPARγ ligands ciglitazone and troglitazone *inhibited* maturation, thus supporting conclusions from the studies mentioned above [137-139]. Another recent study by Hasegawa et al. [142] pointed out that, in addition to PPARγ action, other, as yet unknown, factors are required for changing the fate of progenitor cells into osteoblasts or adipocytes.

An elegant recent study by Wan and coworkers [134] reviewed by Takada and Kato [143] has pointed in the direction that PPARγ plays a role in the promotion of osteoclastogenesis. Deletion of PPARγ in osteoclasts but not in osteoblasts in the Tie2cre-flox mouse model caused mice to develop

osteopetrosis characterized by reduced medullary cavity space and increased bone mass – effects that are due to impaired osteoclast differentiation. This study also showed that mRNA expression of osteoclast genes were suppressed by the PPARγ *antagonists* GW9662 and T0070907 in wild type controls. These findings are of importance when looking at age-related weakening of bone (decreased osteoblastogenesis, increased osteoclastogenesis and increased adipocytes in bone tissue). The same symptoms have been noted after the use of thiazolidinediones in the treatment of Type 2 Diabetes Mellitus [144].

3.3.2 Fatty acids, PPARs and bone physiology

The PPARγ binding site is quite promiscuous, but α-linolenic, linoleic and arachidonic acids as well as the prostaglandin 15-d-PGJ_2 are amongst the most potent natural PPARγ ligands [145, 146]. In addition, the LA peroxidation products 9, 10-epoxyoctadecenoic acid (9,10 EOA), 9,10 dihydroxyoctadecenoic acid (9,10DHOA) and 9-hydroxyoctadecadienoic acid (9-HODE) have also been reported to be ligands for PPARγ2 [144]. Still et al. [147] have reported action of LA on bone via binding to PPARα/δ. Due to its binding to fatty acids, the PPAR family can act as sensor of the cellular metabolic state and due to the existence of various PPAR isoforms, cause promoted transcription of enzymes for either fatty acid oxidation (PPARα) or storage (PPARγ) [148]. In addition, the PPAR family also influences cell growth and differentiation [149].

Since fatty acids and their oxidation products potently regulate PPAR function, one would expect the stated effects of PPARγ in osteoblastic cell types to be regulated by fatty acids. However, only a few studies on the effect of PPARs acting specifically via fatty acids in osteoblast maturation are available at this point in time. In an excellent study, Lecka-Czernik et al. [144] have shown that different linoleic acid metabolites that are PPAR γ2 ligands and have only small structural differences, may direct osteogenic cells into different differentiation pathways; 9,10 DHOA is both antiadipocytic and pre-osteoblastic; 9,10 EOA is antiosteogenic without stimulating adipocyte differentiation whereas 9-HODE (9-hydroxyoctadecadienoic acid) is proadipogenic without affecting osteoblast differentiation. Furthermore, Maurin et al. [150] have shown that DHA, AA and 15dPGJ2 inhibit proliferation (measured with thymidine incorporation studies) of human primary osteoblastic cells via increased expression of PPARγ. Supporting evidence has come from a different direction: a study in calcifying vascular cells, a subpopulation of bovine aortic medial cells that undergo osteoblastic differentiation and form calcified matrix in vitro, has shown that EPA and DHA inhibited osteoblastic differentiation and mineralization. Transient transfection experiments in the same system also showed that DHA also activated PPARγ [151]. Evidence directly concerning the effects of fatty acids on osteoclast function via PPARs is not available currently, but it is to be expected that fatty acids, as ligands of PPARγ, will play a role here. Caution is, however, advised in the interpretation of experiments in which the effects of fatty acids on PPAR are studied, as the recombinant human PPAR ligand binding domain is locked in an activated conformation by endogenous fatty acids [152].

Progressing to a genetic level, Ackert-Bicknell and coworkers [135] have determined a quantitative trait locus (QTL), a cluster of genes that may contribute to a phenotype for bone mineral disease. This QTL contains the gene for PPARγ. Their results have shown that an increase in dietary fat (presumably saturated) has a significant influence on the alleles present for the PPARγ gene in mice, men and women – this may point the direction for future research in this field. Interestingly, this QTL also contains the gene for lipoxygenase (*Alox)* [153], an enzyme that reacts with arachidonic acid to form leukotrienes , lipoxins and various other biologically active metabolites.

It is also possible that PPARα and LCPUFA may interact in bone since PPARα agonists result in elevated bone mass and OPG [154]. Use of a dual PPARα/γ agonist eradicated osteopenic effects of darglitazone, a PPARγ agonist [155]. DHA is a dual ligand for PPARα/γ, whether it acts through this receptor to enhance OPG and regulate osteoclastogenesis remains to be shown. Alternatively it may act to enhance osteoblastogenesis. *In vivo*, both the PPARα/δ agonists linoleic acid and bezafibrate up-regulate osteoblast differentiation as indicated by enhanced number of osteoblast colonies with ALP activity and elevated bone mass by 7-11% [156].

4. Summary and Conclusions

In summary, enriching diets with n-6 and n-3 LCPUFA regulates bone cell maturation and activity, ultimately leading to changes in bone mass. AA preserves MSC numbers through COX-2 mediated synthesis of PGE_2 by

inhibiting apoptosis [70]. DHA and possibly also other n-3 LCPUFA-rich diets may elevate or preserve bone mass through increasing MSC numbers [13] and through enhanced expression of key transcription factors such as Cbfa1 and osterix [33, 92] that enhance differentiation of pre-osteoblasts into mature osteoblasts. Expression of both Cbfa1 and osterix is enhanced by IGF-1, TGF-β1, BMP and moderate amounts of PGE_2 [92], all of which result from diets rich in n-3 LCPUFA. Other mediators of osteoblast maturation include PPARα ligands such as LA [147] and possibly DHA in association with BMP. Since DHA is a weaker ligand for PPARγ [146], more uncommitted MSC are theorized to differentiate into osteoblasts rather than adipocytes. In a paracrine action, mature osteoblasts and other bone marrow cells such as T-cells or fibroblasts enriched with n-3 LCPUFA release less RANKL and reduced OPG [26, 101], resulting in reduced ligand for RANK on immature osteoclasts. This is largely thought to be a manifestation of reduced synthesis of IL-6 and PGE_2 resulting is less ligand for EP4 receptors and suppression of RANKL expression. Within pre-osteoclasts, RANK expression is down-regulated by n-3 LCPUFA diets [102] in association with suppressed TNF-α [102, 115]. Furthermore, such diets inhibit c-FOS and NFκβ within immature osteoclasts and thus modulate RANKL signalling further reducing maturation of the osteoclast [102]. The result is reduction in TRAP along with lower cathepsin K, TNF-α [102] and ultimately osteoclastogenesis. Diets with n-3 LCPUFA also elevate resolvins in bone marrow of rats (Poulsen 2008 Am J Haematol) that are implicated in the TNF-α that would otherwise stimulate maturation of the osteoclast. Conversely PGE_2, the product of AA, is known to enhance RANK expression in pre-osteoclasts [66], possibly leading to

enhanced osteoclastogenesis and bone resorption. Lastly, PGE_2 extends osteoclast lifespan by inhibiting apoptosis [64]. It can be reasoned that apoptosis of OC would be thus more favourable following n-3 enrichment of the diet (Figure 4).

There is ample evidence that n-3 LCPUFA diets are associated with higher bone mass ([11, 157]. Key mechanisms relate to reduction in PGE_2 synthesis along with suppression of inflammatory cytokines [29]. Thus diets enriched in GLA through PGE_1 synthesis, without enhancement of AA, the precursor of PGE_2, also are beneficial to bone [8]. Much of the research has emanated from animal models fed diets with n-3 LCPUFA far in excess of that in the human diet [157]. Nonetheless, randomised clinical trials [39] and cohort studies [38, 41, 44] support an important role for LCPUFA in human bone health. At this time, more data is required to incorporate n-3 LCPUFA into guidelines for the attainment and maintenance of bone mass in humans. While the mechanism continues to unravel, further studies in humans using high n-3 LCPUFA dietary intakes are required to confirm the benefits in bone.

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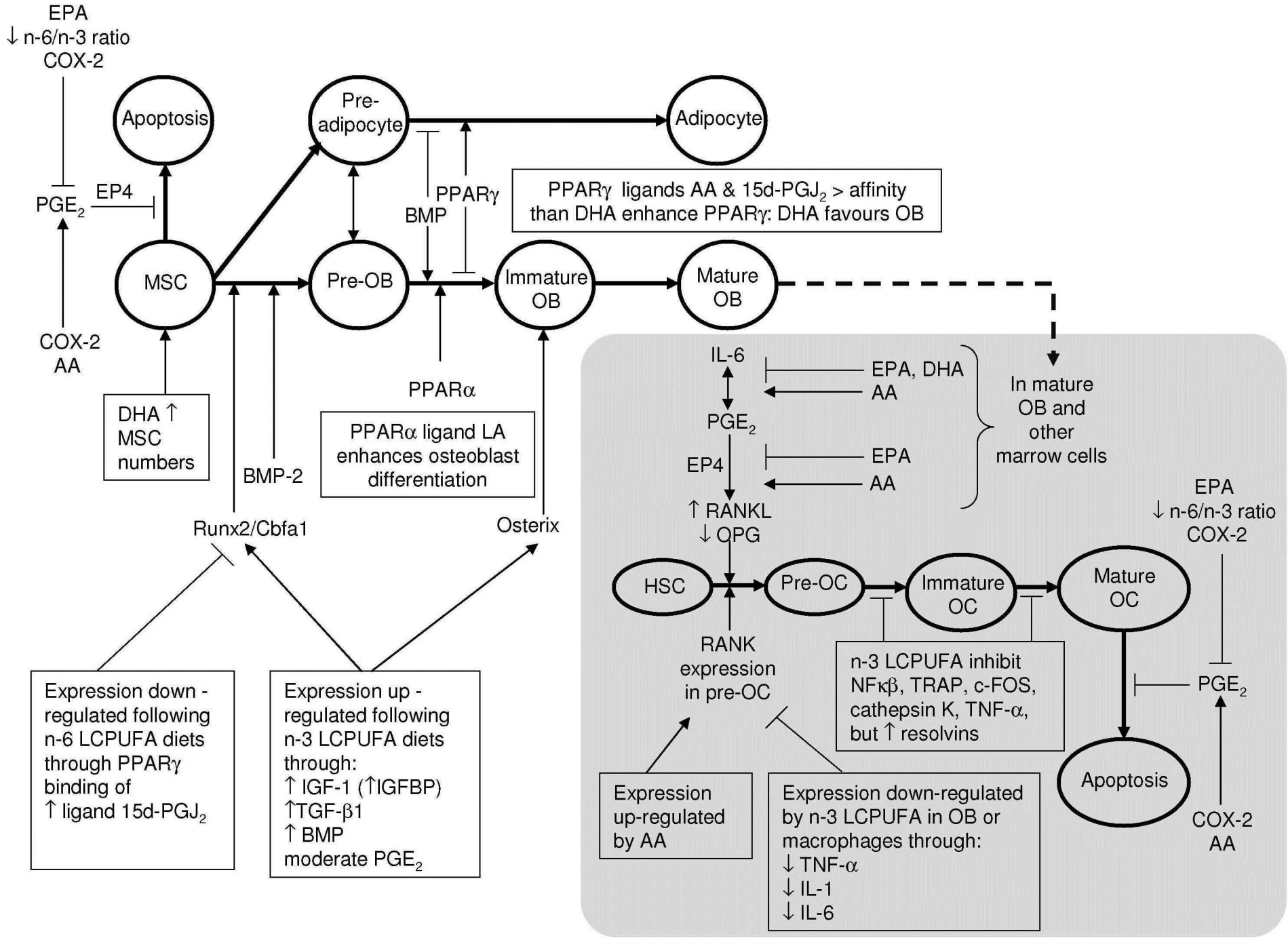
Figure 4: Schematic model of postulated mechanisms by which dietary LCPUFA and cell enrichment leads to mesenchymal stem cell differentiation into mature osteoblasts with cross talk to hematopoietic stem cell differentiation into mature osteoclasts.



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