Long chain polyunsaturated fatty acids and their possible interaction with phytoestrogens: Impact on bone and bone cell function *in vivo* and *in vitro*

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Abstract

Inflammation is a major contributor to postmenopausal bone loss. Various long chain polyunsaturated fatty acids (LCPUFAs), particularly those of the n-3 family, are known to have anti-inflammatory activity and may have a role in minimising postmenopausal bone loss. The objectives of this thesis were to determine whether some LCPUFAs have greater bone-protective effects than others; to identify some of the mechanisms of action of LCPUFAs in bone and to explore the possibility that combined treatment with LCPUFAs and phytoestrogens offers greater bone-protective effects than either treatment alone. Using the ovariectomised rat model for postmenopausal bone loss, the relative effectiveness of eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3) and gamma-linolenic acid (GLA, 18:3n-6) in minimising bone loss post-ovariectomy was investigated. GLA exacerbated bone loss post ovariectomy in rats. I*n vitro,* treatment of MC3T3-E1/4 osteoblast-like cells with GLA resulted in greater membrane-bound RANKL expression suggesting a possible stimulatory effect of GLA on osteoclastogenesis and osteoclast activity. EPA had no effect on overall bone mass *in vivo.* DHA significantly ameliorated ovariectomy-induced bone loss possibly by increasing plasma IGF-1 concentration, modulating vitamin D metabolism and, as observed in a second study, by increasing the concentration of gamma-carboxylated osteocalcin. *In vitro* both EPA and DHA reduced the prostaglandin E2 (PGE2)-induced increase in membrane-bound RANKL expression in MC3T3-E1/4 osteoblast-like cells. However as RANKL-independent pathways are believed to be largely responsible for the ovariectomy-induced increase in osteoclastogenesis *in vivo*, inhibition of RANKL expression may not significantly contribute to the prevention of ovariectomy-induced bone loss. In a second study in ovariectomised rats, combined treatment with DHA and 17β-oestradiol was associated with significantly higher femur bone mineral content than either treatment alone. However, no beneficial effects of combined treatment with DHA and either of the phytoestrogens genistein or daidzein, on bone mass were apparent. *In vitro*, co-treatment of TNF-α – exposed MC3T3-E1/4 cells with DHA and 17β-oestradiol was associated with a higher cell number compared to either treatment alone indicating a protective effect of combined treatment against the cytotoxic and/or anti-proliferative effects of TNF-α. In contrast, combined treatment of MC3T3-E1/4 cells with DHA and genistein, but not daidzein, was associated with significantly lower cell number than either treatment alone. As genistein, but not

daidzein, is a tyrosine kinase inhibitor, this may indicate that DHA requires tyrosine kinase activity for its protective effect on cell number in TNF-α – exposed osteoblasts. Whether DHA itself is bioactive in bone cells or whether lipid mediators formed from DHA are responsible for the observed bone-protective effects is unknown. Using lipid mediator lipidomic analysis, the presence of DHA-derived lipid mediators in bone marrow in quantities known to be physiologically significant in other tissues was confirmed. Further research into the effects of these lipid mediators in bone and confirmation of the mechanisms of action of DHA in bone cells is required. This thesis demonstrates that consumption of DHA provides some protection against ovariectomy-induced bone loss *in vivo* and mitigates the effects of inflammation on RANKL signalling and osteoblast cell number *in vitro*. The bone-protective effects of DHA are complemented by co-treatment with 17β-oestradiol but may be inhibited by co-treatment with the phytoestrogens daidzein or genistein.

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Abbreviations

12-HEPE	12-hydroxy-eicosapentaenoic acid
12-HETE	12-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid
14-HDHA	14-hydroxy-4*Z*,7*Z*,10*Z*,12*E*,16*Z*,19*Z*-docosahexaenoic acid
15-HEPE	15-hydroxy-eicosapentaenoic acid
15-HETE	15-hydroxy-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid
17-HDHA	17-hydroxy-docosa-4*Z*,7*Z*,10*Z*,13*Z*,15*E*,19*Z*-hexaenoic acid
18-HEPE	18-hydroxy-eicosapentaenoic acid
5-HEPE	5-hydroxy-eicosapentaenoic acid
5-HETE	5-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid
AA	Arachidonic acid
ALA	Alpha-linolenic acid
ANOVA	Analysis of variance
ATPase	Adenosine triphosphatase
BA	Bone Area
BMC	Bone Mineral Content
BMD	Bone Mineral Density
BMP	Bone morphogenic protein
BRU	Bone Remodelling Unit
c-AMP	Cyclic adenosine monophosphate
CaR	Calcium receptor
Cbfa1 (RUNX2)	Core binding factor 1
C/EBP-alpha	CAAT enhancer binding protein-alpha
c-GMP	Cyclic guanosine monophosphate
cm	centimetre
COX	Cycloxygenase
CTX	C-terminal telopeptide of type 1 collagen
Dai	Daidzein, 4′,7-Dihydroxyisoflavone 7-Hydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one, 7-Hydroxy-3-(4-hydroxyphenyl)chromone
DEXA	Dual Energy X-Ray Absorptiometry
DGLA	Di-homo-gammalinolenic acid
DHA	Docosahexaenoic acid
DMI	Dry matter intake
DNA	Deoxyribonucleic acid
DPyd	Deoxypyridinoline cross links
ELISA	Enzyme-linked immunosorbent assay
EPA	Eicosapentaenoic acid
eph	Ephrin
ER	(O)estrogen receptor
F	Femur
FCS	Foetal calf serum
FGF	Fibroblast growth factor
g	gram
GC	Gas chromatography
GC/MS	Gas chromatography mass spectrometry

Gen	Genistein, 4′,5,7-Trihydroxyisoflavone 5,7-Dihydroxy-3-(4-hydroxyphenyl)-4H-1-benzopyran-4-one
GLA	Gamma-linolenic acid
HPLC	High performance liquid chromatography
hrs	hours
hrs	Hours
HRT (ERT)	Hormone Replacement Therapy
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
indo	indomethacin
indo	indomethacin
iNOS	Inducible nitric oxide synthase
kg	Kilogram
L	litre
LA	Linoleic acid
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LCPUFA	Long chain polyunsaturated fatty acid
LOX	Lipoxygenase
LRP- 5/6	Low density lipoprotein receptor related proteins 5/6
LS	Lumbar spine
LtB4	Leukotriene B4
LXA4	lipoxin A_4, 5*S*,6*R*,15*S*-trihydroxy-7*E*,9*E*,13*E*,11*Z*-eicosatetraenoic acid
LXB4	lipoxin B_4, 5*S*,14*R*,15*S*-trihydroxyl-7*E*,9*E*,13*E*11*Z*-eicosatetraenoic acid
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage colony stimulating factor
MEM	Minimum essential media
ml	millilitre
mm	millimetre
MMP	Matric metalloproteinase
mol	mole
mRNA	Messenger ribonucleic acid
NADPH	Nicotinamide adenine dinucleotide phosphate
NF κB	Nuclear factor kappa B
NO	Nitric oxide
NPD1, neuroprotectin D1	PD1 generated in neural systems
O-DMA	o-desmethylangolensin
Oes	17β-oestradiol
OPG	osteoprotegerin
Osx	Osterix
OVX	Ovariectomised
PD1, Protectin D1	10*R*,17*S*-dihydroxy-docosa-4*Z*,7*Z*,11*E*,13*E*,15*Z*,19*Z*-hexaenoic acid
PDGF	Platelet derived growth factor
PE	phycoerythrein
PG	Prostaglandin
PPAR	Peroxisome proliferator-activator receptors
pQCT	Peripheral Quantitative Computed Tomography

PTH	Parathyroid hormone
PUFA	Polyunsaturated fatty acid
RANK	Receptor activator of nuclear factor kappa B
RANKL	Receptor activator of nuclear factor kappa B ligand
RBC	Red blood cell
RIA	Radio immunoassay
ROS	Reactive oxygen species
rpm	Revolutions per minute
Rv	Resolvin, resolution phase interaction product
RvD1	7*S*,8*R*,17*S*-trihydroxy-4*Z*,9*E*,11*E*,13*Z*,15*E*,19*Z*-docosahexaenoic acid;
RvE1	5*S*,12*R*,18*R*-trihydroxy-eicosa-6*Z*,8*E*,10*E*,14*Z*,16*E*-pentaenoic acid
RvE2	5*S*,18(*R*/*S*)-dihydroxy-eicosapentaenoic acid
RXR	Retinoid X receptor
s.c.	Sub-cutaneous
Sham	Sham-operated
TGF	Transforming Growth Factor
TNF	Tumour necrosis factor
TRAFs	Tumour necrosis factor receptor activated factors
TRAP	Tartrate resistant acid phosphatase
Tx	Thromboxane
Wnt	Wingless type

Introduction

The age-old cliché "you are what you eat" aptly describes the crucial role diet plays in overall health. In general, there are three possible "fates" for dietary components once ingested. Some are catabolised to provide energy; others are incorporated into structural elements of body tissues. A select few are "bioactive" and involved in the regulation of various metabolic processes. In healthy animals, hormones direct the activity of diet-derived bioactive factors thus ensuring the regulation of metabolic processes is not simply subject to the dietary whims of the individual. However, conditions leading to hormone deficiency, such as menopause, may result in loss of this buffering action and diet, for good or ill, may become an even more important determinant of overall health and well-being.

Post-menopausal osteoporosis is a major cause of morbidity and mortality in developed countries. Although post-menopausal bone loss can be effectively prevented by the use of oestrogen/hormone replacement therapy (ERT or HRT), recent findings from the Women's Health Initiative study indicate such therapy is associated with an increased risk of breast cancer and heart disease. As a result, there is a need to develop low-risk alternative therapies to HRT.

Long-chain polyunsaturated fatty acids (LCPUFAs) and phytoestrogens are bioactive molecules present in the diet. Both epidemiological data and dietary intervention studies have highlighted the possible therapeutic potential of LCPUFAs and phytoestrogens for the prevention or treatment of post-menopausal bone loss. Although the anti-osteoporotic effects of phytoestrogens have received considerable research attention, much less is known about the role of LCPUFAs in preventing postmenopausal bone loss. The main objectives of this thesis were to further knowledge about the role of LCPUFAs in the regulation of bone metabolism. A second objective was to determine if combined treatment with LCPUFAs and phytoestrogens would have greater bone-protective effects than either treatment alone. Modulation of dietary intake of LCPUFAs and phytoestrogens may be a low-risk means of reducing postmenopausal bone loss and consequently, lowering the risk of development of post-menopausal osteoporosis.

CHAPTER 1: Literature Review

Long chain polyunsaturated fatty acids and their possible interaction with phytoestrogens: Impact on bone and bone cell function *in vivo* and *in vitro*

Part 1

Bone Structure and Metabolism: An Overview

Bone is a dynamic tissue. Throughout the lifecycle it is continually broken down and re-built allowing the skeleton to adapt to the changing stresses of life. Aside from its structural role, bone is also a reservoir for calcium, a crucial cell signalling ion, and the home for bone marrow which has critical roles in haematopoiesis and in the immune system. Bone metabolism is tightly regulated by hormones, cytokines and growth factors. Hormonal imbalance and/or chronic changes in cytokine or growth factor activity as a result of life-stage or life-style can drastically affect bone integrity ultimately leading to fracture.

BONE STRUCTURE

Bone is 75% solid and 25% fluid [1]. The solid phase consists of an organic matrix comprised mainly of type-1 collagen on to which calcium, phosphate and trace amounts of other minerals are deposited. The majority of the calcium and phosphate in bone is in the form of a crystalline product known as hydroxyapatite ($Ca_{10}(OH)_2(PO_4)_6$) [2]. Several non-collagenous proteins are also present in the bone matrix such as osteonectin and osteocalcin. The function of these proteins is poorly understood however they may be involved in the process of mineralization.

Bone is enclosed in a dense membrane of fibrous connective tissue known as the periosteum. The periosteum contains blood vessels and nerve endings and is an important means of communication between bone and the rest of the body [3]. Immediately beneath the periosteum lies cortical or compact bone. Cortical bone is highly calcified and provides structural rigidity to the skeleton. Within cortical bone, the bone matrix is arranged in concentric circles or lamellae to form osteons (also known as the Haversian system). Osteons contain haversian canals through which blood vessels flow. The inner surface of cortical bone is known as the endosteal surface [4]. Located within the core or "medullary cavity" of bone is a lattice-like network of bony tissue which is interspersed with hematopoietic bone marrow. This lattice-like bone is less

highly calcified than cortical bone and is referred to as trabecular (or cancellous or "spongy" bone) (**Figure 1**). Because of its lattice-like structure, trabecular bone has a large surface area and is more metabolically active than cortical bone [5].

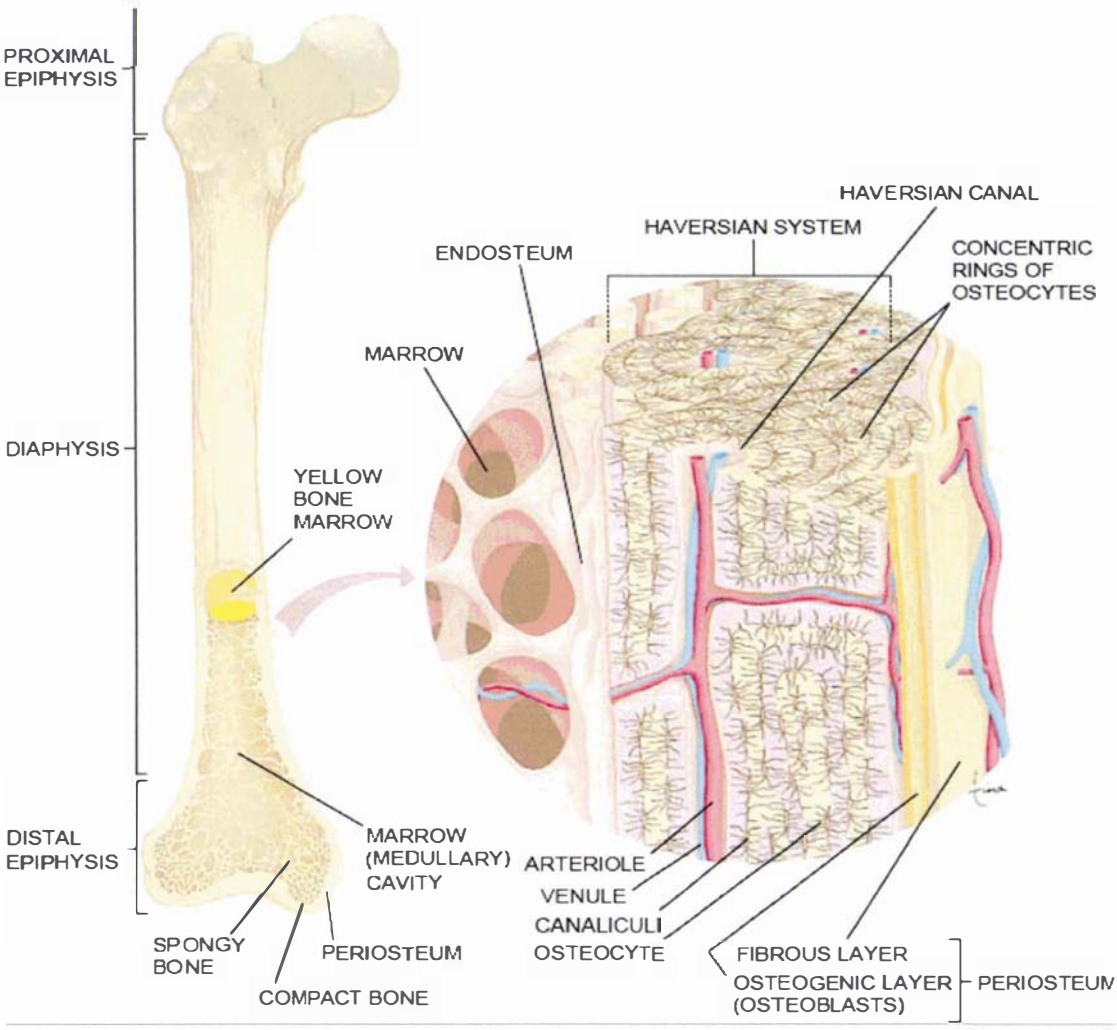

Figure 1 The internal structure of long bones. Bone marrow is interspersed with trabecular bone. Cortical bone (containing the Haversian System) is separated from the rest of the body by the periosteal membrane. Diagram reproduced from Tabers online Cyclopaedic Medical Dictionary, 2004 F.A. Davis Company http://www.tabers20.com/pdf/bone.pdf.

BONE METABOLISM

During childhood and adolescence, bone increases in length as well as density by a process known as bone modelling. Long bone growth ceases in adolescence however, small increases in bone density can occur during early adulthood. Bone density peaks in approximately the third decade of life [6] then begins to decline at an estimated rate of 3% per decade for cortical bone and 7-11% per decade for trabecular [7]. In women the rate of bone loss accelerates to an estimated 9-12% per decade for cortical bone and 13% per decade for trabecular at the time of menopause [8].

Bone remodelling is the method by which bone is continually turned over or renewed. It occurs throughout the lifecycle and involves the sequential and coupled resorption of a small area of bone tissue followed by replacement of this tissue with new bone [9]. In humans, an estimated 10% of bone is remodelled each year [10], the vast majority of which is trabecular bone [11].

Bone remodelling takes place at discrete sites within the skeleton known as Bone Remodelling Units (BRU). Osteocytes, which are cells embedded within bone tissue identify the sites within bone which require remodelling and signal the need for establishment of a new BRU. In humans, bone resorption at a BRU takes approximately 2-3 weeks and is carried out by specialised macrophage-like cells known as osteoclasts. The replacement of resorbed bone with newly synthesised bony tissue requires 3-6 months and is accomplished by a third type of bone cell known as the osteoblast [12, 13].

Although remodelling is generally regarded as the only process governing new bone formation in adults, one other process known as "adult bone modelling" has recently been documented. Adult bone modelling involves new bone formation without prior bone resorption [9]. The quiescent osteoblastic bone-lining cells in the periosteum are directly activated by mechanical loading to secrete new bone matrix immediately on top of existing bone tissue [9]. Adult bone modelling occurs in response to pathological conditions, such as fracture, but has also been shown to occur following bone loading within the normal physiological range [9].

Adult bone modelling results in an increase in bone mass and strengthening of bone tissue [9]. In contrast, bone remodelling results in no net change in bone mass, although localized strengthening of bone tissue may occur due to replacement of damaged or fatigued bone tissue [14].

Bone Cells

There are three main types of bone cell: osteoclasts, osteoblasts and osteocytes.

Osteoclasts

Osteoclasts are multinucleated, hematopoietic cells derived from the monocyte-macrophage lineage. They are responsible for breaking down calcified tissue [15]. Osteoclasts exhibit many activities characteristic of macrophages including the ability to phagocytose cells. They are also activated by several of the same signals which activate macrophages.

Upon exposure to the appropriate range of growth factors, hematopoietic stem cells differentiate into osteoclasts, macrophages or dendritic cells [16]. There are four stages in the formation of active osteoclasts from progenitor cells: commitment of progenitor cells to the osteoclast lineage, differentiation of these osteoclast precursors into mononuclear osteoclasts, fusion of mononuclear cells to form a quiescent multinucleated osteoclast and finally activation of the osteoclast (characterised by attachment to the bone surface) [17]. Macrophage Colony-Stimulating Factor (M-CSF) (produced by osteocytes and osteoblasts) and the transcription factor PU.1 (derived from B lymphocytes and non-lymphoid bone marrow) are essential for the initial proliferation and commitment of progenitor cells to the osteoclast lineage [17-19]. Differentiation, fusion and final activation of osteoclasts is largely controlled by a triad of proteins consisting of two receptors: RANK (Receptor Activator of Nuclear Factor-κB) and OPG (osteoprotegerin), and a ligand, RANKL [17]. M-CSF induces expression of RANK in osteoclast precursors enabling differentiation and maturation into multi-nucleated osteoclasts [17].

RANKL, (also known as TRANCE or ODF), is a member of the tumour necrosis factor (TNF) family [20]. It is expressed by both osteoblasts and osteoclasts as well as by

activated T cells and cells in the liver, spleen, muscle, brain and in the smooth muscle of the arteries [21]. RANKL exists in both membrane-bound and soluble forms [22]. Both the membrane-bound and soluble forms of RANKL interact with the receptors RANK and OPG which compete for RANKL binding. RANK is a membrane-bound receptor whereas OPG is a soluble protein. Both are present in a range of different cell types such as dendritic cells, endothelial cells, fibroblasts, B and T lymphocytes and osteoclasts [21]. Binding of RANKL to RANK and interaction of the cytoplasmic tail of RANK with a family of adaptor proteins known as TRAFs (TNF-associated factors) activates signalling cascades including NF-κB, and MAPK ultimately leading to expression of genes which are essential for osteoclast differentiation [19, 23]. RANKL signalling also helps prevent osteoclast apoptosis [24].

Increased expression of OPG results in increased RANKL/OPG binding at the detriment of RANKL/RANK binding. Binding of RANKL to OPG does not trigger osteoclast differentiation and consequently OPG is often referred to as a decoy receptor [21]. Increased OPG expression leads to a rapid reduction in osteoclast number through both the prevention of new osteoclast differentiation as well as increased apoptosis of mature osteoclasts [21].

Aside from their role in regulating osteoclast differentiation, RANK, RANKL and OPG are also involved in the immune and vascular systems [21]. Mice deficient in OPG not only exhibit decreased bone density and increased risk of fracture but also increased renal and aortic calcification [21]. The kidney and intestine also express high levels of OPG which has led to the suggestion that OPG may have a role in regulating calcium and phosphate balance [17]. Both RANKL and RANK knockout mice fail to develop lymph nodes [21]. In the absence of osteoclasts, a functional bone marrow capable of haematopoiesis fails to form [23]. The immune system also has a role in the regulation of bone resorption as activated B-cells and T-cells express RANKL [18, 25]. Activated T cells also produce several cytokines which upregulate osteoclastogenesis such as TNF-α (Tumour Necrosis Factor-α) and IL-1 (interleukin-1) [18]. In addition, several cytokines such as IL-7 and IFN-γ (interferon-γ) which activate T-cells during the inflammatory response also promote osteoclast activity under certain conditions [18]. Although T-cell activation promotes osteoclastogenesis, when T cells are not exposed to strong activation signals, there is some evidence they may actually suppress bone

resorption [18]. The interaction between the immune and bone regulatory systems has important consequences for human health. Infection, inflammation and auto-immune disorders which lead to activation of the immune system are associated with increased osteoclastic activity and a decline in bone density [18].

Hormones such as 1,25-dihydroxyvitamin D3, parathyroid hormone (PTH) and localised regulators such as TNF-α, prostaglandins (particular PGE2) and various interleukins (IL) influence osteoclastogenesis mainly by altering the balance between RANK and OPG expression and the availability of RANKL as shown in **Figure 2** [15].

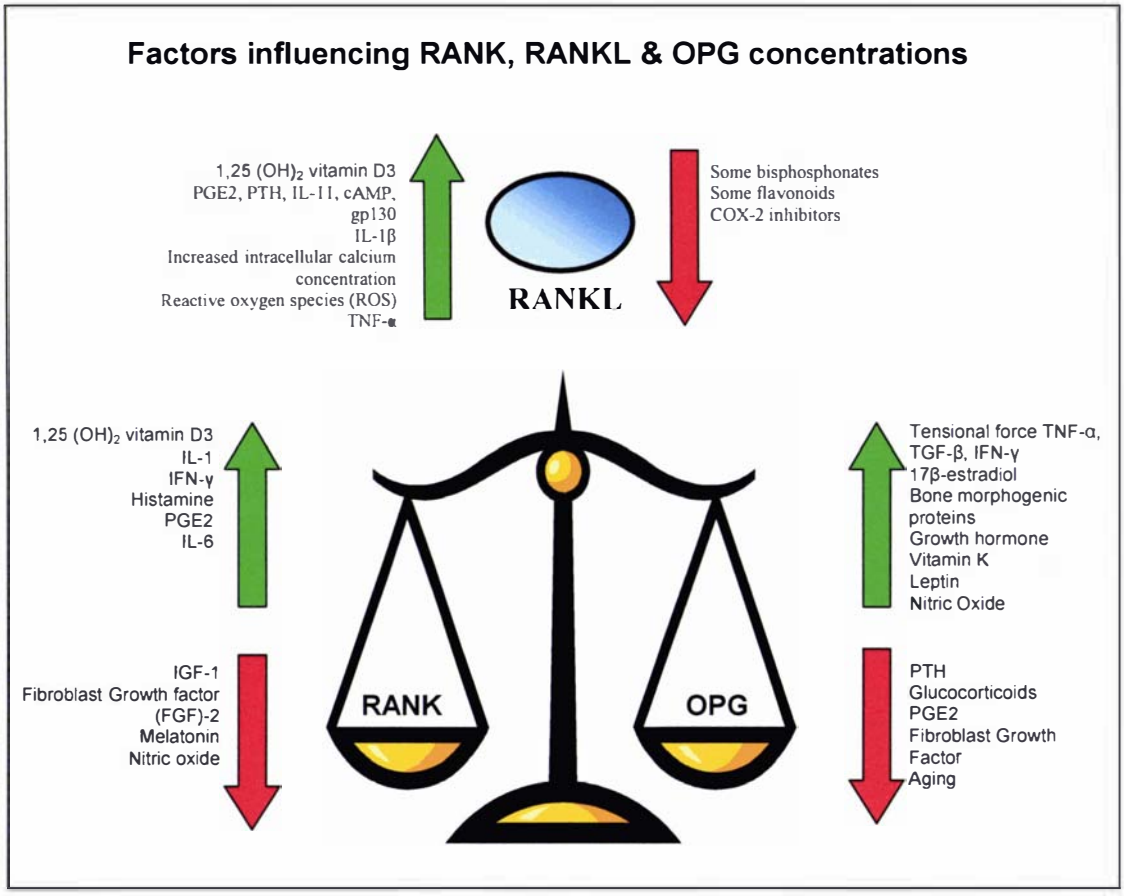

Figure 2 Factors influencing the synthesis of RANK, RANKL and OPG. RANK is influential in the functioning of the immune system and consequently, known immune stimulators such as histamine and inflammatory cytokines, enhance RANK synthesis. Most hormones and localised regulators which are known to have an anabolic effect on bone promote OPG synthesis. Diagram created from information in Theoleyre et al (2004), Turner & Robling (2004,) Nishida et al (2005), Wang et al (2006) and Weitzmann & Pacifici (2005) [18, 21, 26-28].

Although the RANKL/RANK system is vital for osteoclastogenesis, it is not the sole means by which osteoclast differentiation is regulated. For instance, T-cell derived TNF-α is capable of stimulating osteoclastogenesis by increasing stromal cell

production of RANKL [18] as well as by a mechanism independent of the RANK/RANKL pathway as illustrated by the finding that osteoclast formation in $RANK^{(-/-)}$ mice is induced by TNF-α [25]. Other signalling pathways such as Akt, c-Fos and Erk (extracellular signal-regulated kinase) may also be required in conjunction with RANKL/RANK to enable irreversible differentiation of precursors into fully active osteoclasts [19].

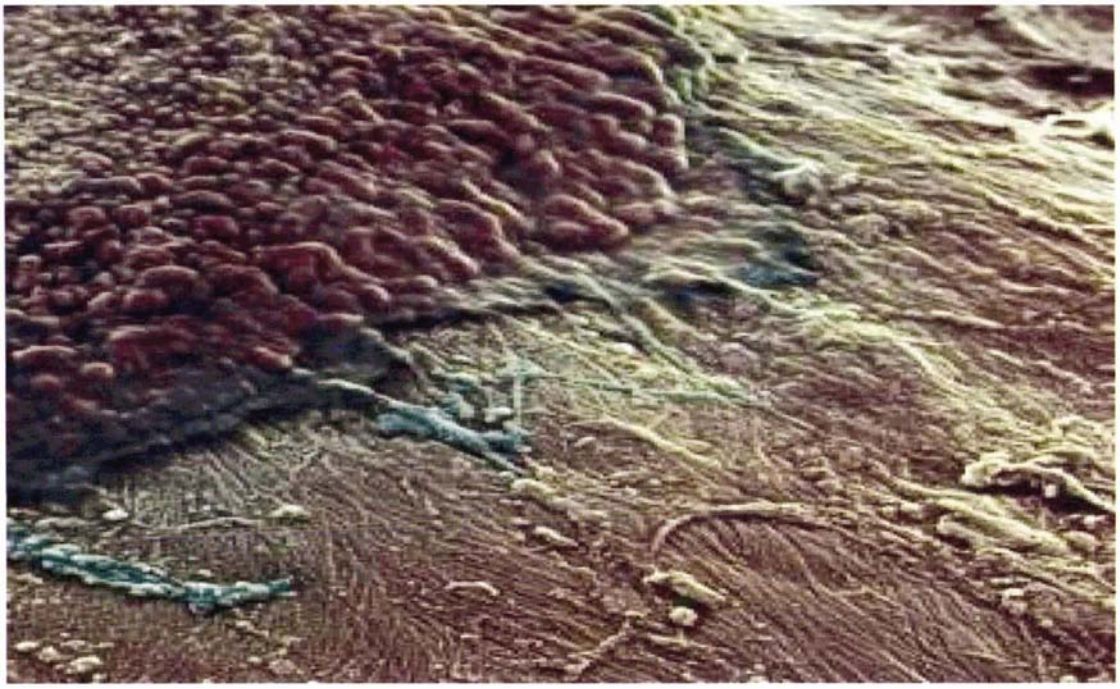

Figure 3 Coloured scanning electron micrograph (SEM) of an osteoclast (upper left) resorbing bone. Magnification: × 1000 at 6 × 7 cm size. Photo courtesy of SPL/Photo Researchers, Inc.

Osteoclast activity

Activation of the differentiated, multinucleated osteoclast occurs by its binding to the bone matrix [17]. This leads to re-organisation of the actin cytoskeleton, polarisation of the cell and the formation of three specialised areas within the plasma membrane of the osteoclast known as the sealing zone, the ruffled border and the functional secretory zone [16, 17].

Microfilaments exuding from the osteoclast form a ring-shaped, "sealing zone" allowing tight adherence of the cell to the bone surface. Integrins (integral membrane proteins) are proteins located in the sealing zone [16]. These interact with matrix metalloproteins (MMPs) (a family of proteins present in the organic bone matrix) and

affix the osteoclast to the bone surface [29]. Within this sealing zone lies the ruffled border which is a convoluted membrane rich in vacuoles. It contains vacuolar H^+-ATPase which acidifies the sealed area dissolving the inorganic constituents of bone [25]. The ruffled border also secretes acidic proteases such as TRAP (tartrate-resistant acid phosphatase) and cysteine proteases which degrade the organic bone matrix [30, 31]. The remnants of degraded bone matrix are absorbed by the osteoclast via endocytosis, transported through the cell and then secreted at the functional secretory domain in the basolateral membrane [16, 17].

Osteoblasts

Osteoblasts are generally described as originating from mesenchymal stem cells [32] located in the bone marrow stroma and the periosteum [33]. These same stem cells also give rise to adipocytes, fibroblasts and chondrocytes [33]. The term "mesenchymal stem cell" however is ambiguous as there are no known markers which distinguish a stem cell as being mesenchymal. Over a decade ago, Chen and colleagues sought to characterise the population of stem cells which gave rise to osteoblasts. They, and since then others, found CD34+ stem cells located within bone marrow can give rise to both hematopoietic progenitor cells as well as osteoblasts [34, 35] suggesting that both osteoclasts and osteoblasts may originate from the same cell source.

In contrast to osteoclasts, the pathway leading to commitment of progenitor cells to the osteoblast lineage is less well understood. Interestingly, there is a great deal of heterogeneity in the mature osteoblast phenotype and considerable variation has been noted in terms of expression of receptors for various cytokines, growth factors and hormones [32, 36].

Two transcription factors have been identified which have important roles in osteoblast differentiation. These are core binding factor-1 (Cbfa-1 also known as RUNX2) and Osterix (Osx).

The essentiality of Cbfa-1 for osteoblast differentiation and bone formation was unequivocally demonstrated in deletion studies in mice. The skeleton of *cbfa-1*-null mice is devoid of bone and comprised only of cartilage [32]. *cbfa-1*–null mice also completely lack both osteoblasts [32] and osteoclasts [37]. Cbfa-1 has an integral role not only in the differentiation of mesenchymal progenitors into pre-osteoblasts and

hypertrophic chondrocytes but also in controlling the function of mature osteoblasts [37]. It regulates the expression of all major osteoblastic genes including OPG and "late-stage" genes such as osteocalcin and controls the rate of formation of new bone matrix [38].

Osx acts downstream of Cbfa-1 to promote the differentiation of pre-osteoblasts into mature osteoblasts [37]. Transcription of *osx* is upregulated by Cbfa-1 [39]. There is a complete absence of osteoblasts in the skeleton of *osx*-null mice however in contrast to cbfa-1-null mice, multinucleated osteoclasts are present [37]. The absence of a functional *osx* gene appears to change the fate of pre-osteoblasts by switching them to the chondrocyte rather than osteoblast phenotype [37]. Several other transcription factors, such as members of the Fos family, operate downstream of Osx and are involved in regulating expression of genes characteristic of mature osteoblasts [12]. Regulation of Cbfa-1 and Osx can occur at the transcriptional, translational and, at least for Cbfa-1, post-translational level [12, 32, 40].

Although the discovery of the activities of Cbfa-1 and Osx has provided considerable insight into the process by which osteoblasts form, much remains unknown about the course of events which leads to initiation of Cbfa-1 and Osx synthesis and activity. Several signalling pathways operate upstream of Cbfa-1 and Osx. The bone morphogenic protein (BMP) and canonical Wnt (Wingless-type) signalling pathways have been identified as having major roles in osteogenesis and are receiving considerable attention at present. In addition, signalling via the three MAP kinase (MAPK) subunits, Erk, Jun and p38, is also important in the regulation of osteoblast differentiation.

BMPs are members of the transforming growth factor superfamily. Four of the more than 20 BMPs currently identified are known to have osteogenic effects. In addition, another member of the transforming growth factor superfamily, transforming growth factor β (TGF-β) also regulates osteoblastogenesis by a similar mechanism to BMPs [41]. BMP-receptor binding activates the intracellular transcription factors known as smads ultimately leading to nuclear translocation of the BMP-smad complex and the initiation of both *cbfa-1* and *osx* gene expression [42-44]. In addition, BMPs also stimulate osteoblastogenesis by smad-independent pathways such as by the activation of

MAPK [45]. For instance signalling by BMP-2 and the jnk and p38 subunits of MAPK stimulates *Osx* expression in osteoblasts [44]. BMPs are also involved in post-translational activation of Cbfa-1 and have been shown to initiate phosphorylation of Cbfa-1 via the MAPK pathway [40].

The Wnt family of glycoproteins activate a number of pathways in different cell types. In bone, osteoblastogenesis is induced by the canonical pathway which involves stabilisation of β-catenin via interaction of Wnt with its receptor Frizzled and co-receptors LRP-5/6 (low density lipoprotein receptor-related proteins 5 and 6). Once stabilised, β-catenin is translocated to the nucleus where it regulates transcription of a number of genes including *cbfa-1* [45] and osteocalcin [46]. Wnt signalling also suppresses C/EBP-alpha (CAAT enhancer binding protein-alpha) and PPAR-gamma, two transcription factors involved in promoting mesenchymal progenitor cell differentiation into adipocytes [47]. The Wnt and BMP signalling pathways do not operate in isolation and there is considerable cross-talk between the two pathways [45].

In the case of Cbfa-1, phosphorylation is required for activation. PTH and growth factors such as fibroblast growth factor-2 (FGF-2) stimulate MAPK-phosphorylation, and therefore activation, of Cbfa-1 [40]. Synthesis of proteins characteristic of the mature osteoblast phenotype such as type-1 collagen, the major constituent in bone matrix and osteopontin, a protein involved in matrix mineralisation, is also triggered by MAPK signalling [48].

Many of the bone regulatory hormones as well as various cytokines and growth factors such as IGF-1 and nitric oxide act on the BMP, Wnt or MAPK signalling pathways as one of the means by which they control bone remodelling [49, 50].

Osteoblast Activity

Osteoblasts attach to the organic bone matrix via interactions between integrins at special focal adhesion sites on the osteoblastic membrane and organic constituents of the bone matrix [26]. Under non-pathological conditions, osteoblasts accurately replace the volume of bone previously resorbed by osteoclasts and the overall effect of bone remodelling is no net change in actual bone mass. The mechanism triggering osteoblasts to stop synthesising new bone matrix once the lacuna is refilled is poorly understood.

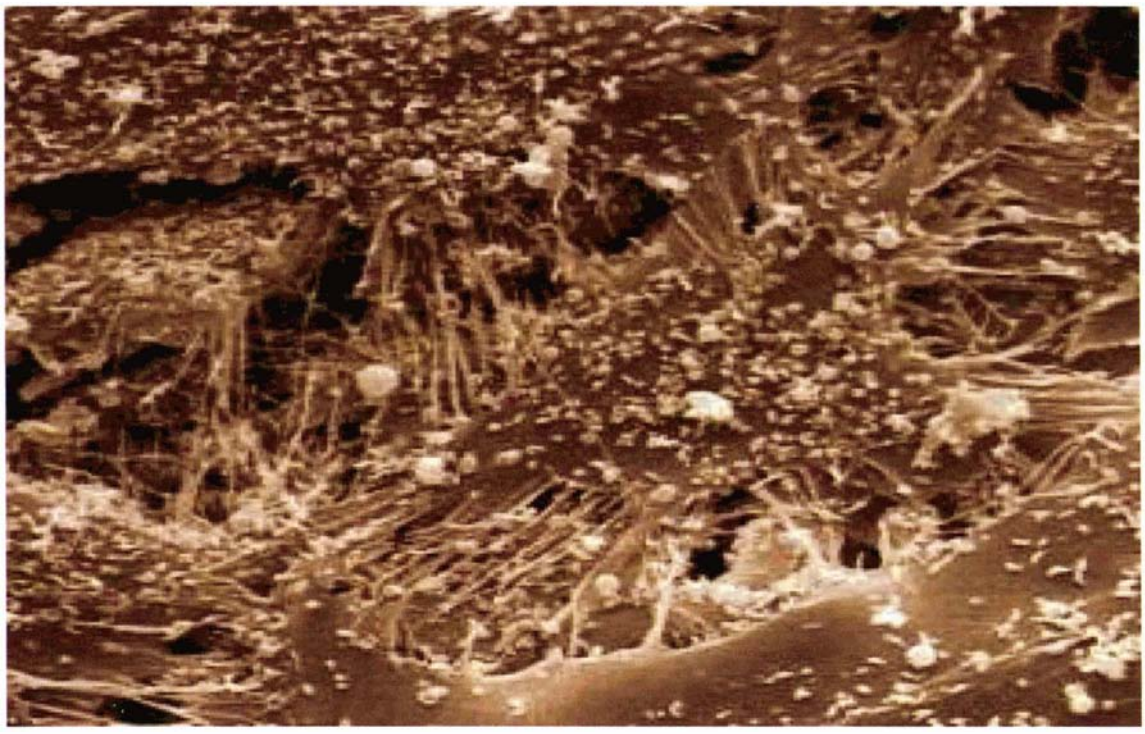

Figure 4 Coloured scanning electron micrograph (SEM) of growing human osteoblasts exuding long strands of extracellular matrix. These strands are used to hold neighbouring osteoblasts together. Photographed at the Imperial College Center for Tissue Engineering, London, UK. Magnification: × 1480 at 5 × 7 cm size. Photo courtesy of Ioanis Xynos / Photo Researchers, Inc.

Osteoblast/Osteoclast Cross-talk

Cross-talk between osteoclasts and osteoblasts is important for initiating both osteoblast and osteoclast formation, maturation and activity [51, 52]. It is also important for ensuring the timely and sequential recruitment and activity of osteoclasts and osteoblasts at a BRU.

Traditionally, "coupling agents" were believed to be involved in osteoclast/osteoblast cross-talk. Members of the TGF superfamily, particularly TGF-β, as well as other growth factors such as IGF-1, IGF-2 and platelet-derived growth factor (PDGF), were proposed as candidate coupling agents [53]. These growth factors are synthesised by osteoblasts and secreted into the bone matrix during new bone formation. They remain embedded in the bone matrix until they are released during osteoclast-mediated bone resorption. The release of these growth factors was thought to stimulate the proliferation of osteoblast precursors and the attachment of new osteoblasts to the remodelling site hence ensuring the lacuna created by the osteoclast was re-filled [53]. However,

although the presence of these factors in bone matrix is indisputable; their role in stimulating osteoblastogenesis during bone resorption now appears to be minor. This is illustrated by the finding that differentiation of ostcoblasts from bone marrow stromal cells is actually enhanced with bisphosphonate therapy, a treatment which prevents osteoclastic bone resorption and therefore prevents the release of matrix-embedded growth factors [54].

There are several examples of cross-talk between osteoclasts and osteoblasts which do appear to have a tangible effect on differentiation or activation of either cell type. One of the best characterised examples is RANKL/OPG/RANK signalling which has a major impact on osteoclastogenesis. In human aortic valves, RANKL also increases Cbfa-1 binding to DNA [55]. Whether RANKL has a similar effect on Cbfa-1 in bone is unknown.

Recently, involvement of the ephrin family of cell surface proteins in controlling both osteoblastogenesis and osteoclastogenesis has been observed [52] and it is possible that ephrins are involved in initiating the transition between bone resorption and formation in the remodelling cycle.

Osteoclasts express a range of ephrins but do not express Eph receptors. In contrast, osteoblasts constitutively express both ephrins and Eph receptors. EphrinB2 (expressed by osteoclasts) can bind to a range of Eph receptors however EphB4 (expressed by osteoblasts) solely interacts with ephrinB2. Binding of ephrinB2 to EphB4 stimulates osteoblastogenesis by inducing *cbfa-1* and *osx* expression. Whether this transcriptional stimulation involves the Wnt or BMP signalling pathways is as yet unknown. Ephrin B2/EphB4 binding also inhibits osteoclastogenesis by a pathway independent of RANKL/OPG signalling [52]. Increased expression of ephrinB2 in either osteoblasts or osteoclasts, or over-expression of EphB4 in osteoblasts, promotes osteoblastogenesis and inhibits osteoclastogenesis. RANKL stimulates ephrin B2 expression however other factors regulating ephrin B2 and Eph B4 expression have yet to be identified [52]. Although artificial manipulation of ephrin B2/EphB4 signalling has been demonstrated to control bone formation and resorption *in vivo* and *in vitro*, whether ephrin signalling has similar effects on bone under normal physiological conditions remains to be

determined. The interaction between osteoblasts and osteoclasts through ephrin and RANKL signalling is illustrated in **Figure 5**.

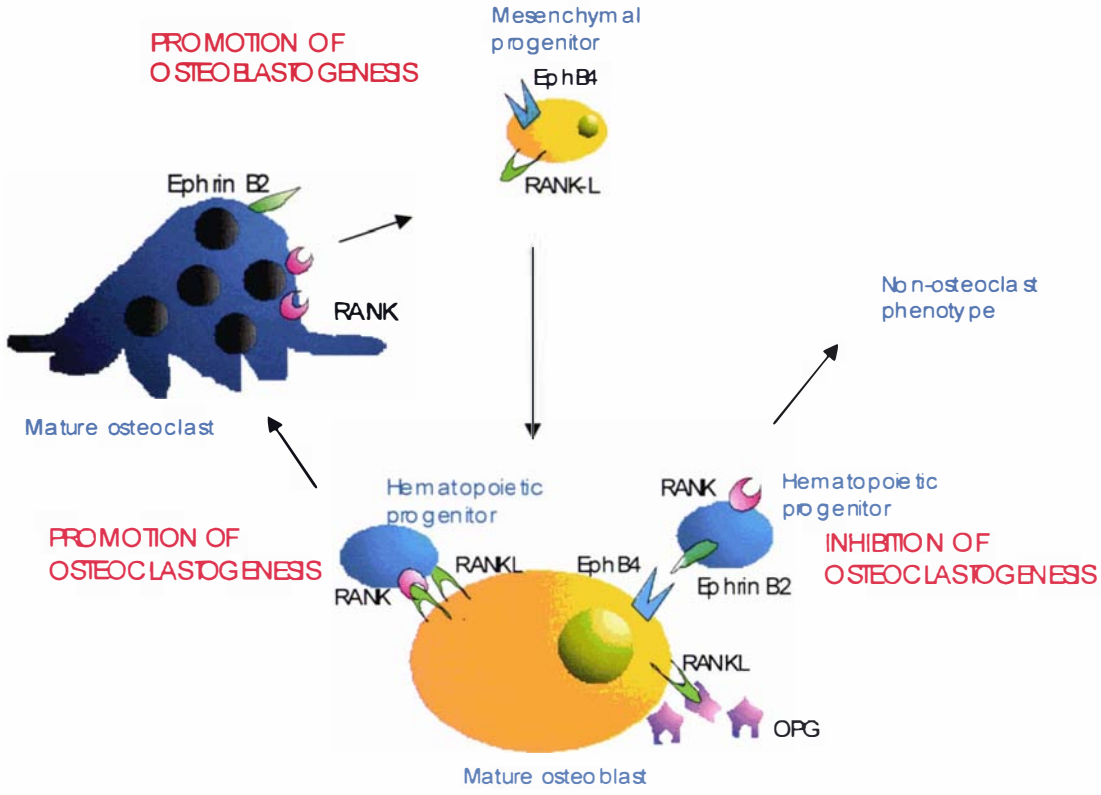

Figure 5 Cartoon representation of examples of osteoblast/osteoclast cross-talk. Promotion of osteoclast maturation is triggered by binding of RANK, on osteoclast progenitors, to RANKL, on osteoblasts. Increased OPG synthesis by osteoblasts results in competitive inhibition of RANKL-RANK binding and inhibition of osteoclastogenesis. In addition, interaction between Ephrin B2 on osteoclasts and the osteoblast EphB4 receptor may also lead to inhibition of osteoclastogenesis and promotion of osteoblastogenesis. Diagram created from information in Zhao *et al* (2006) and Theoleyre *et al* (2004) [21, 52].

Osteocytes

Following new bone formation, some osteoblasts are killed by apoptosis whereas others become embedded within the lacunae in the bone tissue, subsequently transforming into osteocytes [56] (**Figure 6**). Matrix metalloproteinases (MMPs) are involved in preventing osteoblast apoptosis possibly by degrading pro-apoptotic extracellular signalling molecules as well as by activating latent TGF-β enabling osteoblast

differentiation into osteocytes [57]. Part of the osteocyte transformation process involves the production of long, dendritic processes by transforming osteoblasts which extend through the canaliculi in bone and connect to the processes from existing osteocytes [56]. The end result is a 3-dimensional network, referred to as the osteocytic membrane or synctium, which not only connects bone cells but also defines a common fluid space [1]. This synctium also includes osteoblasts and to a lesser extent, osteoclasts, residing on the bone surface, allowing communication between osteocytes in different locations within bone as well as between the three cell types [1].

Osteocytes comprise 90% of total bone cells [56]. Young osteocytes are known as formative osteocytes. They retain many of the same cellular features as osteoblasts [58] and are also capable of bone formation [36]. Young osteocytes are approximately 30% smaller in mean volume than the original osteoblast. They continue to decrease in size, mainly by decreases in the size of the cytoplasm, as the lacuna in which they reside becomes more extensively mineralised [36].

Formative osteocytes mature into resorptive osteocytes which share many features characteristic of osteoclasts such as tartrate-resistant acid phosphatase (TRAP) activity. Resorptive osteocytes can break down bone tissue [58]. The bone resorptive capability of osteocytes is transient however and as the cells age further they lose this capability and are subsequently known as degenerative osteocytes [58].

During bone resorption, osteocytes are released from their lacunae. Some of these released osteocytes are engulfed by osteoclasts. Engulfed osteocytes do not show characteristic signs of necrosis or apoptosis and osteocytes have been detected on the bone surface near BRU. It is possible that osteoclasts engulf embedded osteocytes along with the bone tissue and exude them at the basolateral membrane with other components of the bone matrix [59]. Upon release, these osteocytes may revert to the osteoblast phenotype and synthesise new bone matrix or may become re-embedded in newly formed bone matrix [59]. Cell remnants have also been detected in osteoclasts during both bone modelling and remodelling [59] therefore it appears that whereas some osteocytes "escape" during bone resorption, others are phagocytosed by osteoclasts [60].

Death of osteocytes within bone tissue is typically followed by resorption of the bone matrix in which the osteocyte resides [1]. Although osteocytes are generally long-lived with an average half-life of 25 years [1], various factors can initiate premature programmed cell death [56]. These include bone damage (even at a microscopic level), immobilisation of bone, exposure to cytokines such as tumour necrosis factors or Il-1 and steroid hormone deficiency [56].

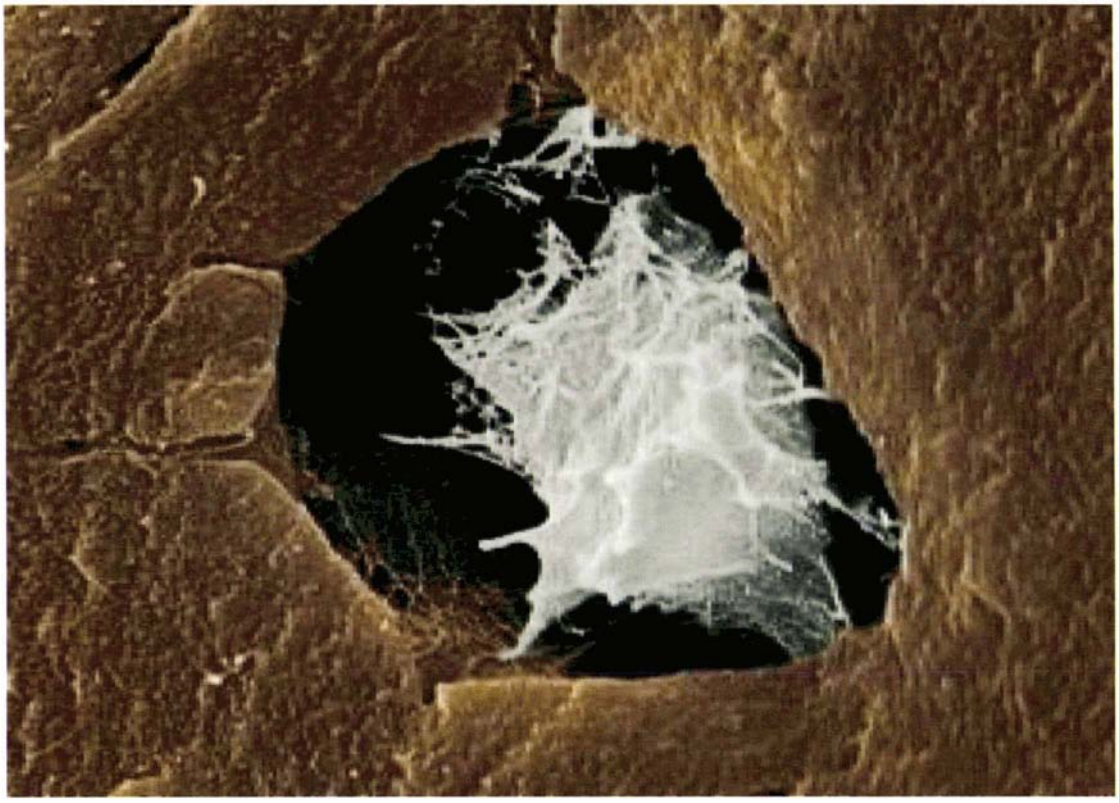

Figure 6 Coloured scanning electron micrograph (SEM) of a freeze-fractured osteocyte surrounded by bone. Magnification: × 4000 at 6 × 7 cm size. Photo courtesy of SPL / Photo Researchers, Inc.

Initiation of Bone Remodelling

Mechanical Strain

Skeletal loading causes localised bone deformation or strain. Osteocytes, as the resident bone cells, are uniquely situated to register the occurrence of strain and to signal the need for bone remodelling [26, 61]. Osteocytes respond to mechanical strain by invoking a biochemical cascade of events ultimately leading to induction of osteoclast activity and a new bone remodelling cycle [62]. In response to bone strain there is a rapid increase in both osteoclast [62] and osteoblast number and activity [63, 64]. The

mechanism by which osteocytes "sense" mechanical strain is poorly understood. As bone deformation results in a change in the flow of bone fluid, fluid shear stress is believed to be one of the factors initiating bone remodelling [62, 63, 65].

Within seconds of bone loading, several locally-acting regulators of bone metabolism are released, primarily by osteocytes, but also by mature osteoblasts. These include nitric oxide (NO) and prostaglandins such as PGE2 [66]. Phospholipase-mediated membrane release of fatty acids, notably arachidonic acid (AA), the substrate for PGE2 synthesis, is also increased in osteocytes as one of the initial responses to mechanical stimulation. Expression of the inducible form of COX, COX-2, which oxidises AA to form PGE2, is also upregulated as an early response to strain [62]. PGE2 promotes *cbfa-1* gene expression [67, 68] and stimulates production of IGF-1 and c-AMP. IGF-1 up-regulates *osx* transcription [44] thereby promoting osteoblastogenesis. PGE2 also stimulates osteoclastogenesis by upregulating RANKL and RANK and down-regulating OPG synthesis [69]. Osteocytes subjected to strain synthesise and secrete M-CSF and RANKL [70] thereby enhancing osteoclastogenesis.

The Wnt/β-catenin signalling pathway is activated in both osteocytes and osteoblasts in response to mechanical strain [71] and is believed to have a major role in governing the behaviour of bone cells following skeletal loading [66]. PGE2 is one of the activators of the Wnt signalling pathway [66]. Synthesis of sclerostin, an osteocyte-specific protein which inhibits Wnt signalling, is also decreased in osteocytes in response to mechanical loading [66]. Both BMP and MAPK signalling are activated in response to strain [72].

Bone experiences varying degrees of strain at various times however bone remodelling is only initiated once the level of strain reaches a certain threshold. This threshold or "mechanostat" is determined by the relative levels of systemic hormones [65]. Oestrogen is particularly important for establishing the strain threshold set-point. High oestrogen levels reduce the degree of strain required in order to initiate endocortical bone remodelling and this effect appears to be predominately mediated through Oestrogen Receptor-α (ERα) [65, 73]. In contrast, oestrogen inhibits the ability of mechanical strain to enhance periosteal bone formation, possibly by a mechanism involving ER-β [74]. Parathyroid hormone (PTH) and vitamin D also influence the

setpoint of the mechanical strain threshold [56, 75]. The concentrations of these bone-active hormones are influenced by the overall calcium balance within the body.

Calcium Balance

Dietary calcium intake has a major impact on bone mass as it is the main source of calcium for bone mineralisation. Factors influencing dietary calcium intake, intestinal calcium absorption and renal calcium resorption determine overall calcium balance in the body.

Calcium is an important second messenger. Intracellular calcium concentration can rapidly increase by up to 100-fold as part of the calcium-signalling process [76]. Extra-cellular calcium concentration however is maintained within a very narrow range (1.1-1.3mM). A specialised, G protein-coupled calcium-sensing receptor (CaR) expressed on cell membranes "senses" extracellular calcium concentration and modulates the synthesis and secretion of systemic hormones accordingly [77, 78].

Acute changes in calcium balance do not invoke bone remodelling. Such fluctuations are buffered by the "exchangeable calcium pool" present within bone fluid. Approximately 25% of bone is fluid and an estimated 1% of total body calcium ("the exchangeable calcium pool") is contained within bone fluid [1]. Bone fluid circulates within the canicular system and is separated from plasma and extracellular fluid by the synctium [1]. The exchangeable calcium pool present in bone fluid is the primary source for replenishing extracellular calcium levels and the "sink" for excess extracellular calcium. The buffering effect of the exchangeable calcium pool means that small fluctuations in extracellular calcium concentration can be rectified without the need for inciting changes in bone cell activity. Chronic changes in extracellular calcium balance however will trigger a hormonal cascade ultimately leading to a shift in the set-point of the bone resorption/formation balance [53].

The CaR is capable of modulating the secretion of PTH, calcitonin, growth hormone, gastrin and insulin as well as several other peptides which also regulate intestinal calcium absorption, renal calcium reabsorption and/or bone cell genesis and activity [78]. In response to elevated extracellular calcium concentration, the CaR also activates

phospholipase-C and stimulates the release of fatty acids from membranes [78] and induces MAPK signalling via the Jnk subunit thereby promoting osteoblast proliferation [79].

Regulation of Bone Remodelling

A neuro-endocrine pathway governing both total bone and fat mass through leptin binding to hypothalamic receptors has been identified [80]. Other systemic regulators of bone metabolism include insulin, growth hormone and oestrogen which promote bone formation; thyroid hormone and glucocorticoids which stimulate bone resorption; calcitonin which inhibits bone resorption and PTH and 1,25-$(OH)_2$-vitamin D3 which stimulate both bone formation and resorption [81].

Whilst growth hormone has a major role in promoting bone growth during childhood, a triad of hormones, consisting of oestrogen, parathyroid hormone and vitamin D, is largely responsible for responding to calcium-sensing and mechano-sensing signals in adulthood.

In the gastrointestinal tract and in osteoblasts parathyroid hormone (PTH), in conjunction with 1,25-dihydroxyvitamin D enhance transcription of the 24-hydroxylase gene thereby stimulating further 1,25-dihydroxyvitamin D formation. In the kidney however, PTH and 1,25-dihydroxyvitamin D3 have opposing effects on 24-hydroxylase mRNA levels [82]. Oestrogen promotes 1,25-dihydroxyvitamin D activity by increasing vitamin D receptor (VDR) expression [83, 84]. 1,25-dihydroxyvitamin D3 inhibits PTH synthesis thereby indirectly acting as a feedback inhibitor of its own synthesis [82]. Oestrogen reduces PTH receptor number in some cells consequently reducing cell responsiveness to PTH [85] but it also enhances PTH gene expression [86].

PTH, 1,25-dihydroxyvitamin D and oestrogen are not the sole systemic regulators of bone metabolism. A change in the levels of these three hormones also influences levels of other bone-active hormones. 1,25-dihydroxyvitamin D and possibly, oestrogen, may enhance calcitonin secretion particularly in postmenopausal women [87]. Calcitonin raises 1,25-dihydroxyvitamin D levels and inhibits bone resorption by suppressing

osteoclast activity. $1,25(OH)_2$ vitamin D in conjunction with serum calcium, may enhance pancreatic secretion of insulin [88] which is essential for bone mineralisation.

Regulation of Calcium Balance

Intestinal calcium absorption is promoted, and renal calcium reabsorption inhibited by 1,25-dihydroxyvitamin D. PTH increases overall calcium balance by promoting reabsorption of calcium from the kidneys rather than through any direct effect on intestinal calcium absorption. PTH also stimulates the release of calcium from the exchangeable calcium pool in bone fluid [53]. Oestrogen indirectly enhances intestinal calcium absorption by increasing intestinal cell responsiveness to vitamin D [83, 84]. It is unknown whether oestrogen also directly enhances intestinal calcium absorption however in the kidneys, oestrogen increases PTH activity and reduces urinary calcium excretion [89].

Regulation of Bone Cell Formation and Function

PTH, oestrogen and vitamin D act both synergistically and antagonistically to control the synthesis and activity of osteoblasts and osteoclasts.

The effect of PTH on bone cells is complex. Low intermittent doses of PTH promote collagen synthesis and bone formation [53] whereas high, continuous PTH release inhibits collagen synthesis, stimulates collagen breakdown and promotes osteoclastic bone resorption [82]. *In vitro,* PTH has a biphasic effect on osteoclasts, first increasing activity of existing osteoclasts, then promoting new osteoclast differentiation [82]. It is somewhat unclear to what extent PTH acts directly on osteoclasts or how much of its effect is indirect and a consequence of its action on osteoblasts [82].

The effects of PTH on osteoblasts are many and varied. PTH may disrupt osteoblast-binding to the bone surface, therefore allowing the replacement of osteoblasts with osteoclasts. The anabolic effects of PTH on bone may at least partially be mediated via the Wnt and/or TGF-β/BMP signalling pathways [90]. PTH decreases the activity of mature osteoblasts, but is mitogenic to pre-osteoblasts and may promote new osteoblast

formation [82]. PTH inhibits transcription of *cbfa*-1 and *osx* [91] but promotes phosphorylation and therefore activation of Cbfa-1 protein [40].

The effect of oestrogen on osteoclasts is species specific. Oestrogens directly affect avian osteoclast activity however their effects on rat osteoclasts appear to be largely indirect [89]. Human osteoclasts express both oestrogen receptors (ER-α and ER-β) [92] therefore it is possible that oestrogen acts directly on human osteoclasts. Oestrogen interferes with the catabolic effects of PTH in promoting bone resorption but does not appear to hinder the anabolic activity of PTH [89]. Oestrogen also stimulates bone formation in trabecular bone [93]. It enhances the activity of Cbfa-1 by both an ER-α – dependent and independent mechanism but does not appear to affect Cbfa-1 expression [94]. Conversely Cbfa-1 suppresses oestrogen activity. ER-α dimerises with Cbfa-1 and this dimerisation is perhaps involved in the mechanism by which oestrogen increases Cbfa-1 activity [94]. ER-α also dimerises with β-catenin and this interaction is markedly increased by binding of oestrogen to ER-α. The relevance of this interaction to bone is unknown however studies in drosophila indicate synergistic promotion of β-catenin–mediated gene expression by ligand-bound ER-α In osteoblasts, estrogens activate BMP-signalling by stimulating transcription of various BMPs including BMP-2 [95].

Whereas PTH and oestrogen work to change the balance between bone resorption and bone formation, the actions of 1,25-dihydroxyvitamin D are a little more indiscriminate. 1,25-dihydroxyvitamin D promotes formation of both osteoblasts and osteoclasts [96, 97].

Hormones can act directly on target cells to bring about change however the majority of the effects of hormones are brought about by the action of localised regulators. Hormone-receptor binding leads to an increase in gene expression or translation of specific localised regulators. Known localised regulators of bone metabolism include the eicosanoids, cytokines and growth factors which directly affect osteoclast or osteoblast proliferation, differentiation or activation [98, 99]. **Figure 7** illustrates some of the interactions between the major regulatory pathways controlling bone remodelling.

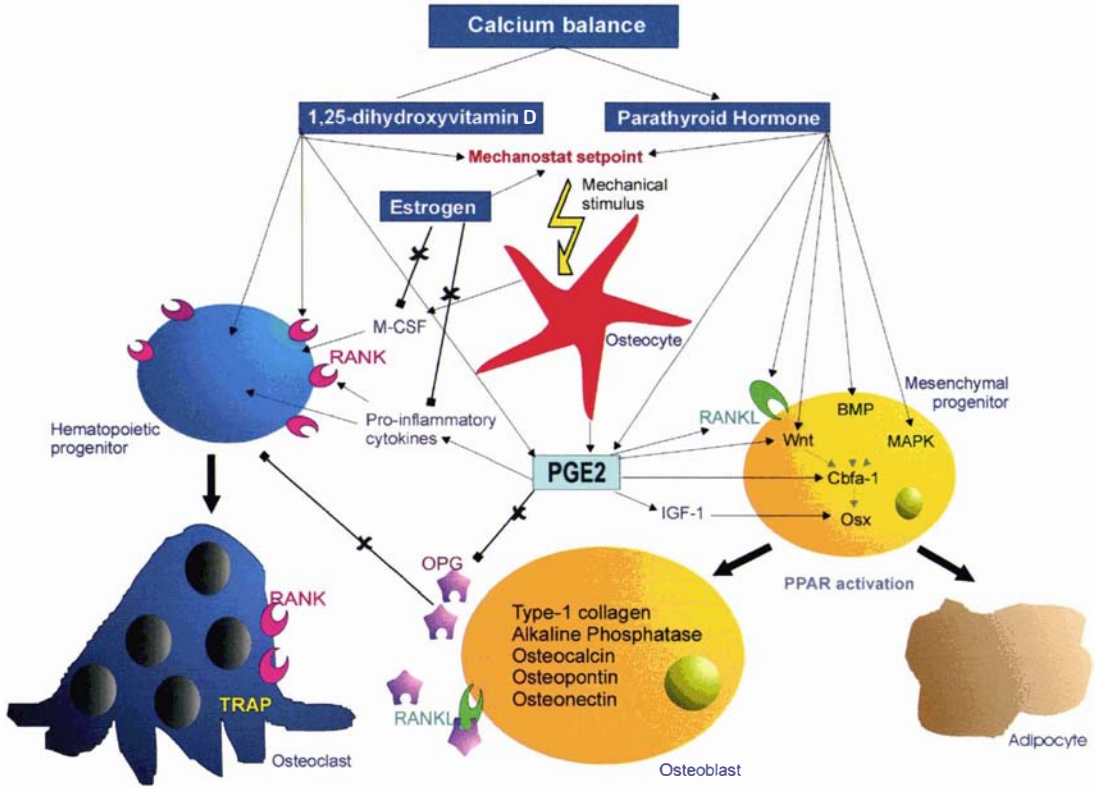

Figure 7 Summary diagram of some of the key regulatory mechanisms governing osteoblast and osteoclast formation and differentiation. The central role of PGE2 in this process is highlighted. Diagram created from information in Celil *et al*, 2005; Celil & Campbell, 2005; Tobimatsu *et al*, 2006; van der Horst *et al*, 2005; Franceschi *et al*, 2003; Bonewald, 2006; Smith & Clark, 2005; Yoshida *et al*, 2002 and Watkins *et al*, 2003 [44, 62, 66-68, 90, 100, 101]

M-CSF = Macrophage colony stimulating factor, TRAP = Tartrate Resistant Acid Phosphatase, OPG = Osteoprotegerin, Cbfa-1 = Core binding factor 1, BMP = Bone Morphogenic Protein, MAPK = Mitogen Activated Protein Kinase, Wnt = Wingless type (canonical signalling pathway), PPAR = Peroxisome Proliferator-Activator Receptor, IGF-1 = Insulin-like growth factor-1, RANK = Receptor activator of nuclear factor kappa B, RANKL = Receptor activator of nuclear factor kappa B ligand.

Misregulation of Bone Remodelling: Osteoporosis

Osteoporosis is defined as a disease characterized by decreased bone mass, microarchitectural deterioration of bone tissue and an increased risk of fracture [102]. More than 50% of New Zealand women and nearly one third of New Zealand men over the age of 60 years, have osteoporosis. The burden on the public health system is high. Osteoporosis frequently leads to hospitalization, usually as a result of hip fracture. Statistics from several European countries implicate osteoporosis as responsible for the highest number of hospital days in women aged over 45 years [103].

The consequences of osteoporosis are greater than simply an increased risk of bone fracture. Approximately one third of hip fracture patients die within the next year, usually as a result of cardiovascular disease. There is a strong, inverse relationship between degree of aortic calcification and bone density [104]. Coincidentally, cardiovascular disease is linked with increased calcification of the arteries whereas osteoporosis results in decreased calcification of bone tissue [105, 106].

Causes of Osteoporosis

Osteoporosis is categorised into three types each stemming from a different primary causes:

1. *Senescence.* Endocrine changes associated with ageing, in particular, decreased production of growth hormone and insulin-like growth factor–1 (IGF-1), lead to decreased bone formation and therefore a loss of bone mass [6].

2. *Sex hormone deficiency.* The most common cause of sex hormone deficiency is menopause. For this reason, osteoporosis stemming from sex hormone deficiency is usually a result of a lack of oestrogen and is termed postmenopausal osteoporosis. Sex hormone deficiency however can also result from surgical procedures such as castration in males and ovariectomy in females.

3. *Glucocorticoid excess.* Glucocorticoids inhibit intestinal calcium absorption, promote PTH synthesis and inhibit oestrogen synthesis. They also stimulate osteocytes to enlarge the lacunae within which they reside by resorbing bone mineral [31] Whilst the endogenous glucocorticoid, cortisol, is essential for the formation and action of both osteoblasts and osteoclasts, high levels of glucocorticoids lead to a reduction in bone mass [107].

In many cases these three "causes" of osteoporosis do not occur in isolation. For instance, although sex hormone deficiency as a result of natural menopause may be the primary cause of osteoporosis in an individual, age-related decreases in growth hormone production may also contribute to bone loss. Expression of RANKL by T cells also appears to increase with aging [23] which may result in increased osteoclastogenesis and contribute to bone mineral loss. In addition, feedback control of cortisol secretion declines with age. Although the amount of cortisol secreted in response to a stimulus is

unchanged with age, the return to baseline levels after a challenge is noticeably longer in older, compared to younger, animals as well as people [108]. Glucocorticoids are also used in pharmaceuticals commonly prescribed for arthritis sufferers. A side effect of treatment is accelerated bone resorption [107].

Structural changes in bone associated with postmenopausal osteoporosis

Osteoporosis is associated with a reduction in bone mineral density as well as a change in internal bone structure. Changes in the structure and organization of collagen fibres within bone occur with both increasing age as well as with osteoporosis. These changes also have important consequences in terms of the biomechanical properties of bone. To illustrate this, one study compared the risk of fracture in two groups of women, one "young" (average age 45 years) and one "older" (average age 75 years), with the same bone density. The risk of fracture was found to be 7% in the older women compared to just 1% in the younger women [109].

In avian bones, increased lysine hydroxylation and changes in cross-links between collagen fibres is a characteristic of osteoporosis [110]. Crosslinks between collagen fibres are formed by both enzymatic and non-enzymatic means. Whilst there is one report of age-related changes in the pyridinium cross-links (hydroxylysylpyridinoline and lysylpyridinoline) in humans, most studies have found no change in the occurrence of enzymatic cross-links with increasing age [111]. In contrast, an age-related decrease in pentosidine crosslinks (non-enzymatic crosslinks formed via the Maillard reaction) has been reported in humans [111].

An increase in periosteal apposition occurs following oestrogen deficiency however the endosteum is continually eroded and endosteal bone formation is inhibited. This results in an increase in bone circumference but a decrease in cortical bone thickness. It is more marked with advancing age and in females rather than males [74]. Trabeculae become disconnected and more widely spaced as shown in the microtomograph in **Figure 8**.

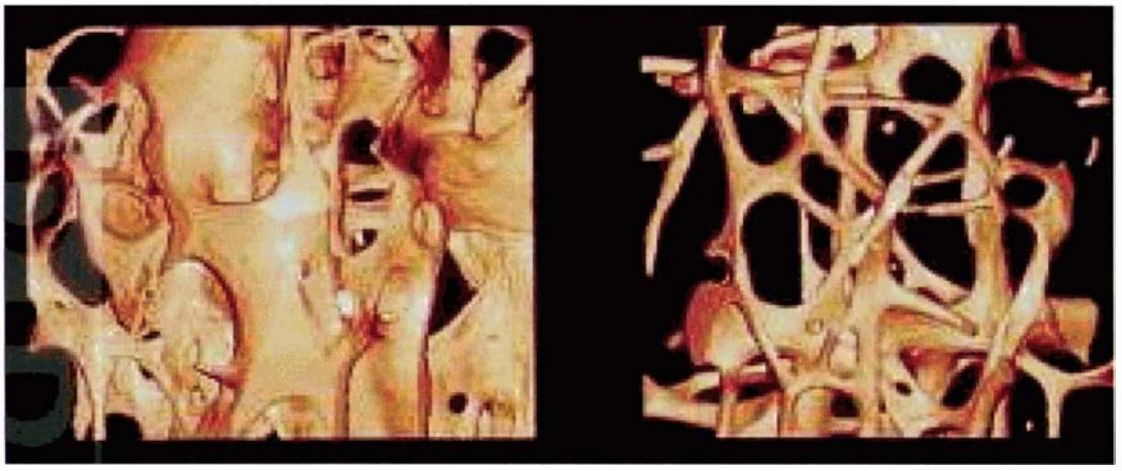

Figure 8 Structure of the vertebrae of a healthy 50 year old (left) and an osteoporotic 70 year old (right) as visualised by x-ray microtomography. Erosion of the trabecular bone has resulted in enlargement of the spaces between trabeculae. Photo courtesy of ESRF-CREATIS / Photo Researchers, Inc.

Metabolic changes associated with postmenopausal osteoporosis

Ovariectomy in rats leads to a selective reduction in vitamin D receptor (VDR) number in jejunal but not renal cells [112]. A reduction in jejunal VDR number results in reduced responsiveness of intestinal cells to vitamin D signalling and therefore reduced intestinal calcium absorption. As no change in renal VDR number occurs, vitamin D stimulation of renal calcium excretion is not impaired. A lack of VDRs may also influence levels of other osteogenic hormones as mice genetically modified to have a non-functioning VDR but with normal serum calcium and phosphorus levels were found to have impaired oral glucose tolerance and insulin secretion [113].

Through interaction with oestrogen receptors (ER), oestrogen controls the synthesis of a number of cytokines and growth factors. Two main types of ER have been identified: ERα and ERβ, both of which are expressed by osteoblasts. Interaction of oestrogen with ERβ results in decreased expression of various inflammatory cytokines particularly interleukin-1 (IL-1), IL-6 and tumour necrosis factor – α (TNF-α). In the case of IL-6, oestrogen inhibits expression by preventing binding of NF-κB to the IL-6 gene. There is some evidence that oestrogen deficiency not only leads to increased levels of inflammatory cytokines but also modulates IL-6 signaling pathways thereby leading to enhanced cell-responsiveness to IL-6 [114].

Aside from direct effects on inflammatory cytokine synthesis resulting from oestrogen-receptor binding, pro-inflammatory cytokine expression is also increased by free radical reactions normally inhibited by the antioxidant activity of oestrogen [114]. Oestrogen deficiency is associated with a gain in adipose tissue [115]. There is evidence that adipose tissue may be a major determinant of circulating IL-6 levels [114].

T-cell derived production of TNF-α is also increased with oestrogen deficiency due to both an increase in T-cell number and activation [18]

Oestrogen deficiency is therefore associated with an increase in the overall degree of inflammation. As a result, postmenopausal osteoporosis is considered to have a strong inflammatory component in its aetiology [116, 117]. The series of events triggered by menopause which can lead to the development of osteoporosis are outlined in **Figure 9**.

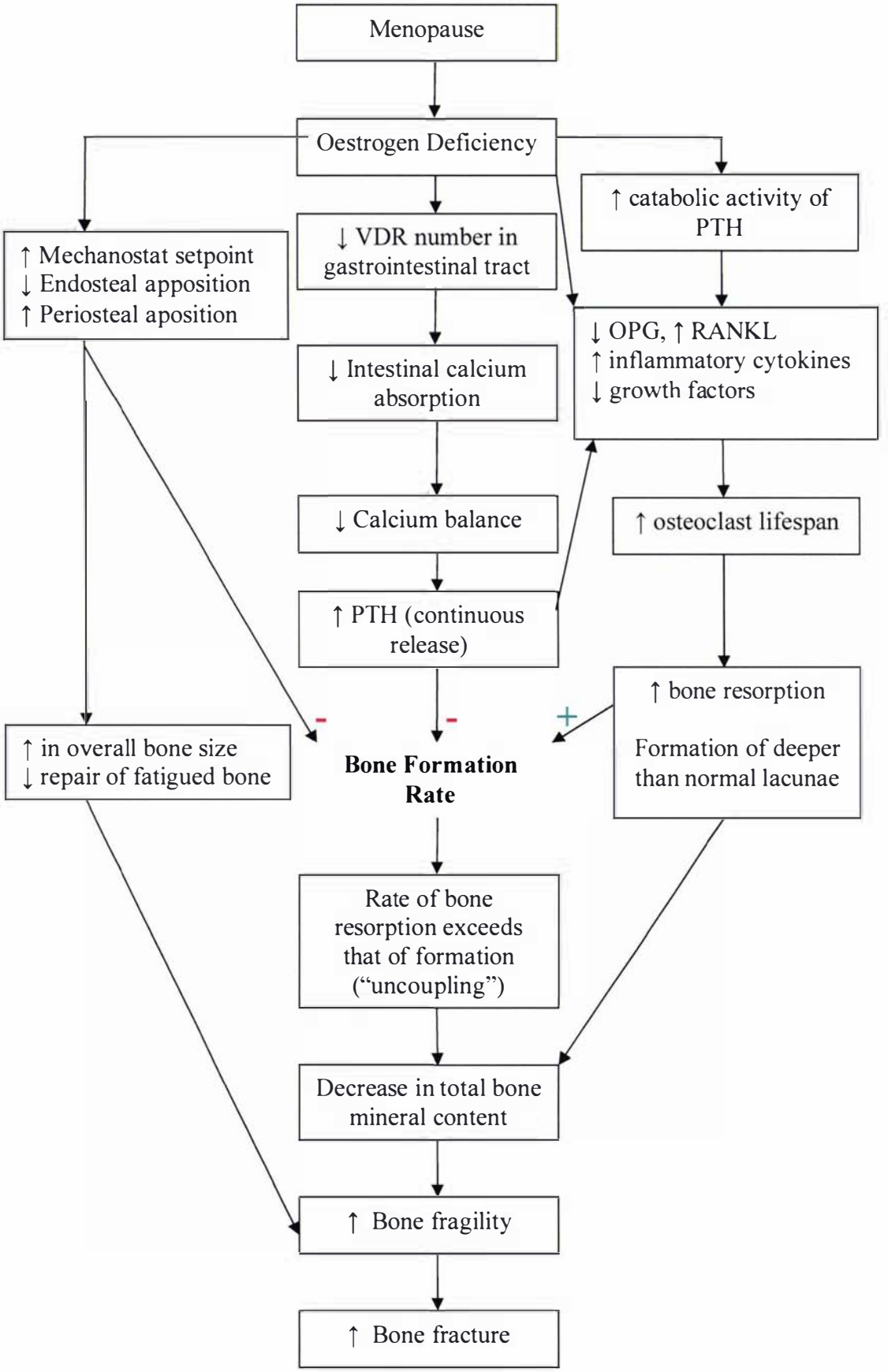

Figure 9 Summary diagram showing the chain of events following menopause which ultimately can lead to increased bone fracture risk. Diagram created from information in Pfeilschifter *et al*, 2002, Hui *et al,* 1988 and Saxon & Turner, 2005 [74, 109, 114].

Part 2:

Long chain polyunsaturated fatty acids and the regulation of bone metabolism

Published as a Mini-Review: Poulsen RC, Moughan PJ, Kruger MC Long chain polyunsaturated fatty acids and the regulation of bone metabolism Experimental Biology and Medicine 2007 232:1275-1288

Although the importance of PGE2 in regulating bone remodelling is well-established, the involvement of LCPUFAs and other lipid mediators in the control of bone metabolism may be much more extensive than is currently recognised. The role of prostaglandins in bone biology has been comprehensively reviewed elsewhere [98, 118]. The present review focuses on the actions of LCPUFAs themselves on bone as well as the effects of their non-prostanoid bioactive metabolites.

LONG CHAIN POLYUNSATURATED FATTY ACID METABOLISM

LCPUFAs are fatty acids with a minimum chain length of 18 carbons containing at least two double bonds. LCPUFAs are classified into one of two 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. As 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 although ALA is present in plant chloroplasts 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 [119] 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 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. 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 [120]. A schematic diagram of LCPUFA metabolism is shown in **Figure 10**.

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 catalyses the conversion of AA to thromboxane A2 (TxA2) [121] and in conjunction with aspirin, the mono-hydroxylation of DHA to form 13R- and 17R-hydroxylated DHA (13R- and 17R-HDHA) [122]. To date, two distinct *cox* genes have been identified encoding two isoforms of COX known as COX-1 and COX-2 [123]. 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 [124]. Due to the smaller size of the substrate binding site of COX-1 compared to COX-2, 22-carbon DHA can only be metabolized by COX-2 [122].

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 [125] possibly by modulating toll-like receptor signalling pathways [126].

The existence of a third isoform of COX has been hypothesised [127] 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 [128, 129].

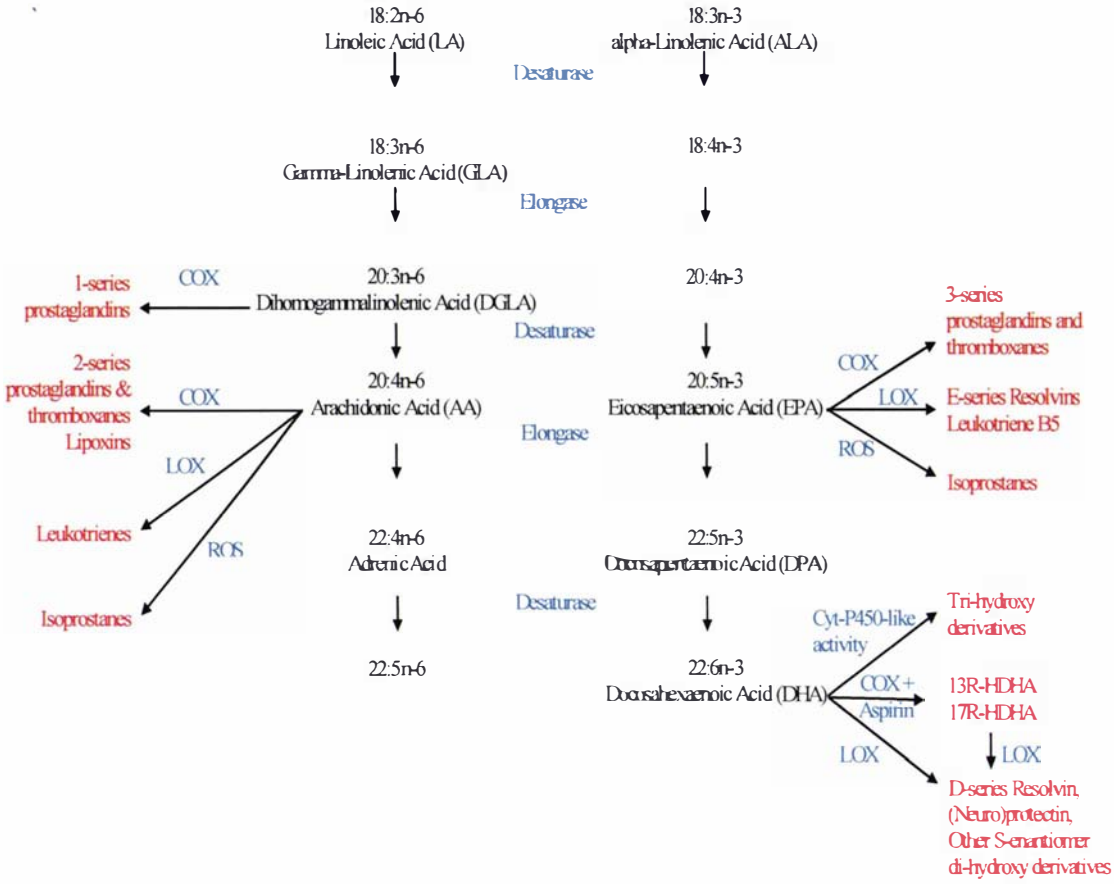

Figure 10 Metabolism of long-chain polyunsaturated fatty acids. LA and ALA are progressively desaturated and elongated by a shared desaturase/elongase enzyme system to form longer-chain and more highly unsaturated fatty acids. Diagram created from information in van Papendorp *et al* (1995), Serhan *et al* (2002), Serhan (2005), Kuhn & O'Donnell (2006) and Shen & Tai (1998) [120-122, 130, 131].

Lipoxygenase and Epoxygenase

There are several isoforms of the LOX enzyme which exhibit differing substrate specificities. 12/15-LOX activity results in generation of hydroperoxy derivatives of 20-carbon LCPUFAs. These can be subjected to further LOX activity resulting in formation of leukotrienes, lipoxins and hepoxilins [131]. 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. [120, 122]. LCPUFAs may also serve as substrates for cytochrome P450-catalysed reactions as trihydroxy DHA derivatives are generated by cytochrome P450-like activity [120, 122].

Non-enzymatic Oxidation

Non-enzymatic metabolism of LCPUFAs also occurs. The isoprostanes are highly oxidized LCPUFA metabolites formed by free radical catalysed oxidation of usually membrane-bound AA, EPA and DHA [132].

Many of the LCPUFA metabolites generated either by enzymatic or non-enzymatic 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 [133], red blood cell (RBC) membranes [134] serum [135] and plasma [136].

Dietary LCPUFA deficiency in animals and humans results in decreased intestinal calcium absorption [137], reduced synthesis of bone connective tissue matrix and loss of cartilage [137], bone demineralisation [138], increased renal and arterial calcification [139], replacement of bone with adipose tissue [138] and severe osteoporosis [139]. 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 [138]. Although a negative association between total LCPUFA intake and bone mineral density (BMD) was observed in one study in post-menopausal women [140], a more recent study that examined dietary intake of the two families of LCPUFAs reported that post-menopausal women with a high dietary ratio of n-6:n-3 fatty acids had the lowest bone mineral density [141]. 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 [142]. 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 post-menopausal women have yielded mixed results. In elderly (mean age 80 years) osteoporotic women, daily supplementation with 4g 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, 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 to supplementation with olive oil, evening primrose oil or fish oil alone [130]. Osteocalcin is a bone-specific protein which 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 [143] and are therefore biochemical markers of bone formation. The results from this study are therefore ambiguous as 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 to all other groups [130]. 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 following the ingestion of n-3 fatty acids or fish oil [144, 145] therefore the increased urinary calcium excretion observed in elderly women following 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 6g of LCPUFA-rich oil (3.6g LA, 480mg GLA, 240mg EPA and 180mg DHA) in conjunction with 600mg calcium carbonate per day for 18 months. Controls who received 600mg of calcium carbonate and 6g 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 [137].

However two subsequent studies showed no effect of LCPUFA supplementation on bone post-menopause. In one study, pre and post-menopausal women (age range 25-40yrs and 50-65yrs respectively) were supplemented with Efacal®, a Scotia Pharmaceuticals product containing a combination of evening primrose oil (4.0g/day providing approximately 430mg GLA/day), fish oil (440mg/day providing approximately 70mg EPA/day) and calcium (1.0g/day) for a period of 12 months. No additional benefit of LCPUFA supplementation on total body BMD over calcium supplementation alone was observed [146]. 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 anti-osteoporotic agents on bone over the relatively short study period. Generally, effects of anti-osteoporotic 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 [5].

In another study menopausal women (age range 45-65 years) receiving 40g 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 to women supplemented with a wheat germ placebo [147]. 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 [141]. 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 [148]. Consumption of a n-6 LCPUFA-rich diet appears to inhibit ALA elongation and desaturation as conversion of ALA to EPA and DHA was reduced by 40-50% when a n-6 LCPUFA-rich diet was consumed [148]. 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 two trials reporting no effect on bone mass which may mean that the very long chain n-3 PUFAs have a beneficial effect on bone post-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-ovariectomised, mature-ovariectomised 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.

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, area and thickness and significantly lower trabecular BMD compared to controls. Serum levels of IGF-1 and leptin were significantly lower in rats whilst on the LCPUFA deficient diet, but returned to normal levels once a LCPUFA-adequate diet was fed [149]. 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 [81, 150]. In piglets, low levels (0.6g/100g fat) of a supplement containing AA and DHA (5:1) increased bone mass; however higher doses (1.2g/100g fat and 2.4g/100g fat) were less beneficial [151]. Similarly, both PGE2 injection or supplementation with AA (0.60% - 0.75% total fat) and DHA (0.1% total fat) increased bone mineral content in male piglets [152, 153] however a combination of the two

treatments resulted in decreased femur BMC [153]. 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 has an anabolic effect on bone mass [154]. At high concentrations, PGE2 promotes bone resorption [155]. In other studies, limiting PGE2 synthesis has been implicated as a means of optimising bone mass. For instance in one-month old Japanese quail n-3 LCPUFA supplementation 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 [156].

Not only is the total amount of LCPUFA in the diet important for optimising bone formation but the composition of dietary LCPUFAs also appears to be important. Many studies have focussed on determining the optimal ratio of n-6:n-3 fats in the diet for maximising bone mass. Watkins *et al* (2000) reported that a ratio of 1.2:1 n-6:n-3 LCPUFAs resulted in a higher rate of bone formation during growth compared to ratios of 23.8:1, 9.8:1 and 2.6:1 [157]. Similarly Green *et al* (2004) reported greater BMD in male weanling rats fed a diet containing a 1.4:1 ratio of n-6:n-3 compared to those receiving a 7.1:1 ratio [158]. Another study in weanling rats found a 3:1 ratio of GLA:EPA resulted in a lower rate of bone resorption [159] and greater overall calcium balance and bone calcium content, compared to a 1:3 ratio [145].

In young animals, both n-3 and n-6 fats appear to be required for bone growth. However much remains unknown about the effects on bone of individual LCPUFAs within the two 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 maximising bone calcium content [134]. High plasma DHA concentration was also associated with lower bone resorption rate in growing piglets [160].

Studies in ovariectomised animals

Supplementation of ovariectomised mice with flaxseed oil (a source of ALA) had no effect on bone mass or bone strength [161]. However increased intake of the very long chain n-3 PUFAs, EPA and DHA, with [162] or without [133, 163] the n-6 LCPUFA

GLA, has been shown to reduce bone resorption [133, 162], inhibit RANKL and inflammatory cytokine synthesis [163] and preserve BMC [133, 162, 163] in ovariectomised 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 [124]. Watkins *et al*, 2006 observed that a ratio of 5:1 n-6:n-3 LCPUFAs was more beneficial than a 10:1 ratio in maintaining bone mass post-ovariectomy in rats regardless of the total dietary PUFA content [133]. However lumbar spine BMC was preserved in ovariectomised mice fed a diet containing a n-6:n-3 LCPUFA ratio of approximately 1:12 [163] and Kruger *et al*, 1999 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 [164]. 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 ovariectomised rat model was used by Watkins *et al,* 2006, whereas Kruger *et al,* 1999 used 6-month old skeletally-mature ovariectomised rats. The LCPUFA requirement to optimise bone mass during bone modelling may differ from that required to optimise bone mass during bone remodelling.

As with studies in non-ovariectomised, growing animals, there is some evidence that different LCPUFAs within the two LCPUFA families may have differing effects on bone in ovariectomised 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 anti-resorptive effect [162].

Most studies have utilised supplements containing a mixture of LCPUFAs, however two studies have examined the effects of EPA alone on bone in ovariectomised rats. Ovariectomy-induced bone loss was prevented by supplementation of ovariectomised rats receiving a low calcium diet (0.01% calcium) with 160mg of EPA/kg body weight/day. However, no effect of EPA was seen in rats receiving a calcium adequate diet [165]. In a second study, 100mg EPA/kg body weight/day had no effect on bone mass whereas 1000mg/kg body weight/day increased the rate of bone resorption and

had a detrimental effect on lumbar spine and femur BMC in ovariectomised rats receiving a calcium-adequate diet [136]. Findings from these two studies suggest that EPA may only be beneficial for preserving bone mass post-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 post-ovariectomy [166]. However a growing ovariectomised rat model was used in this study. Therefore, whether DHA would be more effective than EPA in maintaining BMC post-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 to non-treated and EPA-treated membranes [144]. 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 [167] and decreased faecal calcium excretion [159, 168]. It is possible that physiological changes brought about by increased membrane content of LCPUFAs lead 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 as an increase in overall calcium balance has only been observed with fish oil supplementation [159, 168].

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 [168]. Membrane LCPUFA content is known to affect the structure, fluidity and polarity of membranes as well as the relative proportion of membrane-bound proteins [169]. Structurally, membranes are composed of "liquid disordered" phospholipid regions interspersed with tightly-packed,

more orderly "lipid rafts" which consist of sphingolipids and cholesterol. Cholesterol is essential for the formation of lipid rafts [170]. 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 [169]. However the DHA metabolite 10,17S-docosatriene (protectin D1) has been shown to promote lipid raft clustering in peripheral blood mononuclear cells [171]. Therefore both the LCPUFA content as well as 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 three 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 [170]. 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 [170]. Examples of lipid raft-associated proteins include Ca2+-ATPase [172] and components of the NF-κB kinase complex [173]. Recently an oestrogen receptor-like protein similar to ER-α has also been detected within lipid rafts on the plasma membrane of osteoblasts [174]. 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 [175]. The greatest increase occurs with the addition of two and three double bonds with little change in fluidity occurring with more than three double bonds [169]. 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 [169].

Membrane permeability and the speed of membrane flip-flop (movement of membrane constituents between layers in the membrane bilayer) is increased as the number of double bonds in the fatty acyl chains increases [176]. Increased membrane unsaturation may expedite cellular uptake of nutrients and other molecules particularly by passive transport.

Studies in marine-dwelling bacteria which have the ability to synthesise 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 [175]. 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) [177]. 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 [177]. LCPUFAs as well as prostaglandins and various LOX-generated LCPUFA metabolites are natural PPAR ligands [131, 178, 179]. To date three PPARs have been identified: PPAR-α, PPAR-γ and PPAR- β/δ [178, 180] although at least two subforms of PPAR- γ exist [181]. All three PPARs are expressed by osteoblasts and activation of PPAR-α, PPAR-δ or PPAR- γ1 in pre-osteoblasts can promote differentiation into mature osteoblasts [178]. In contrast, ligand-mediated activation of PPAR-γ2, promotes differentiation of mesenchymal progenitors into adipocytes rather than osteoblasts [178, 182]. 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 [181]. DHA and AA are also believed to be PPAR-γ ligands [179] 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 [183]. This may indicate a positive effect of the two LCPUFAs on osteoblastogenesis as cessation of proliferation and cell cycle withdrawal are characteristic preparative steps for differentiation into the mature osteoblast phenotype [179]. In support of this, an increase in alkaline phosphatase activity (a marker of the mature osteoblast phenotype) in MC3T3-E1 osteoblast-like cells following treatment with n-3 fatty acids has been reported [68]. In hepatocytes, the LCPUFA metabolites HETE and PGJ(2) promote PPAR-α and PPAR-γ expression [184] raising the possibility that these metabolites may also induce PPAR expression in osteoblast precursors.

Fluid shear stress in osteocytes [185] or exposure of osteoblasts to 17β-estradiol or 1,25-dihydroxyvitamin D3 [186] results in a rapid increase in intracellular calcium concentration. Phospholipase, which catalyses the release of AA and DHA from membrane phospholipids [187], is essential for the rise in intracellular Ca2+ resulting from 17β-estradiol or 1,25-dihydroxyvitamin D3 stimulation [186] and may also be involved in increasing intracellular Ca2+ in response to fluid shear [185]. Prostaglandins and other oxidised LCPUFA derivatives are ionophores [188, 189] 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 which inhibit osteoblastogenesis. Some n-3 LCPUFAs inhibit 5-LOX activity and non-enzymatic 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* [190]. The isoprostane 8-isoprostaglandin E2, a product of non-enzymatic oxidation of AA, promotes osteoclastogenesis [191] and inhibits osteoblastogenesis in bone but induces osteoblastic differentiation of vascular cells hence promoting arterial calcification [192]. Supplementation with fish oil or EPA has been demonstrated to reduce deposition of calcium in kidneys and the aorta [139] 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 to upregulation of BMP-4 and retinoid X receptor-α (RXR-α) [193] suggesting LCPUFAs may activate the BMP signalling 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 such as genistein but not by inhibition of COX suggesting it is not a result of PGE2 activity [194]. Nitric oxide (NO) stimulates bone formation and suppresses bone resorption. At high concentrations however, NO inhibits both bone formation and resorption [195].

Effect on Osteoclastogenesis and Osteoclast Activity

AA treatment of MC3T3-E1 pre-osteoblast - like cells resulted in increased secretion of soluble RANKL and decreased secretion of OPG probably as a result of PGE2 activity [196]. Compared to LA and AA, both DHA and EPA decreased osteoclastogenesis and osteoclast activation in bone marrow cell culture [163]. The effects of n-3 LCPUFAs on bone are largely attributed to their inhibitory effect on COX-mediated synthesis of pro-inflammatory prostaglandins (particularly PGE2). PGE3 derived from EPA has similar potency and bioactivity to PGE2 [197], however EPA is believed to be a less efficient substrate for COX and/or an inhibitor of COX activity [198]. Synthesis of pro-inflammatory 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 pro-inflammatory cytokine and PGE2 secretion and osteoclast formation [199]. 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 pro-inflammatory cytokine synthesis [200] and induce osteoclastogenesis by a RANKL–independent mechanism [201, 202]. LtB4 (derived from AA) is generally more potent than LtB5 [203] although the effects of LtB5 on bone cells have yet to be fully investigated. Lipoxins are synthesised by bone marrow cells and have been shown to inhibit some of the actions of leukotrienes [204]. In murine models of inflammation, lipoxins are potent endogenous anti-inflammatory mediators [205]. Their role in the regulation of bone remodelling is largely unknown however topical application of LxA4 in rabbits reduced tissue inflammation and bone loss associated with periodontitis [206] 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 [122, 206, 207]. 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 summarised in **Table 1**.

Table 1 *Summary of known bioactivity of LCPUFAs and their metabolites on calcium balance and bone metabolism.*

	Functions in bone
Arachidonic Acid	Increases intestinal calcium uptake [167] Promotes osteoblastogenesis possibly by activating PPAR-γ [179, 183, 208] Increases inducible nitric oxide synthase (iNOS) expression in osteoblasts [194]
Prostaglandin E2	Biphasic effect – in low concentrations promotes osteoblastogenesis, in high concentrations promotes bone resorption [154, 155] Increases RANKL and decreases OPG secretion by osteoblasts [196]
Leukotriene B4	Inhibits osteoblast activity [190, 200, 201] Promotes osteoclastogenesis and osteoclast activity [200-202]
Isoprostanes	Inhibits osteoblastogenesis & promotes osteoclastogenesis in bone [191, 192] Induces osteoblastic differentiation of vascular cells [192]
Lipoxin A4	Inhibits osteoclastic bone resorption [206]
Eicosapentaenoic Acid	Increases intestinal calcium uptake [167] Decreases osteoclastogenesis and osteoclast activity [163]
Prostaglandin E3	Similar effects and potency as PGE2 [197]
Leukotriene B5	Generally less potent than LtB4 in other tissue systems [203]. Effects in bone unclear.
Resolvin E1	Decreases osteoclastogenesis [199]
Resolvin E2	Bioactive effects in bone are unknown
Docosahexaenoic Acid	Increases intestinal Ca^{2+}-ATPase activity [144] Increases intestinal calcium uptake [167] Promotes osteoblastogenesis possibly by activating PPAR-γ [179, 183, 208] Decreases osteoclastogenesis and osteoclast activity [163]
Protectin D1	Bioactive effects in bone are unknown
D-series Resolvins	Bioactive effects in bone are unknown

An increased need for LCPUFAs post-menopause?

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 [138, 139]. 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 [209]. Changes in serum phospholipid LCPUFA concentrations are also evident following menopause [210] and recent epidemiological evidence suggests that the fatty acid composition of

biological membranes alters following menopause [141]. 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 to premenopausal, breast cancer patients with the greatest differences being evident in membrane content of palmitic acid, oleic acid, LA and DHA [211]. DHA concentrations in serum have been found to be higher in women compared to men [212] and both AA and DHA concentrations were higher in women treated with HRT or the selective oestrogen receptor modulator (SERM) raloxifene, compared to untreated women [213]. Oestrogen may increase the synthesis of AA and DHA from their precursors [212, 213]. Levels of LA and ALA also decline with age in women, and to a lesser extent, in men [209]. 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 pre-formed, very-long-chain PUFAs may be necessary to compensate for the decrease in endogenous LCPUFA synthesis and availability.

Part 3

Phytoestrogens alone and in combination with long chain polyunsaturated fatty acids: Impact on the Regulation of Bone Metabolism

Phytoestrogens are compounds produced by some plant species which exert similar, albeit weaker, effects to animal oestrogens. As shown in **Figure 11**, phytoestrogens have steroid-like structures and are highly stable due to the presence of phenolic compounds at both ends of the molecule [214]. The phenolic ring allows binding to the oestrogen receptor [215].

Several families of molecules are classified as phytoestrogens including lignans, isoflavones and some flavonoids. The most common dietary source of lignans is linseed (also known as flaxseed) whereas soy is a major source of isoflavones. The focus of this review is on two soy isoflavones: genistein and daidzein.

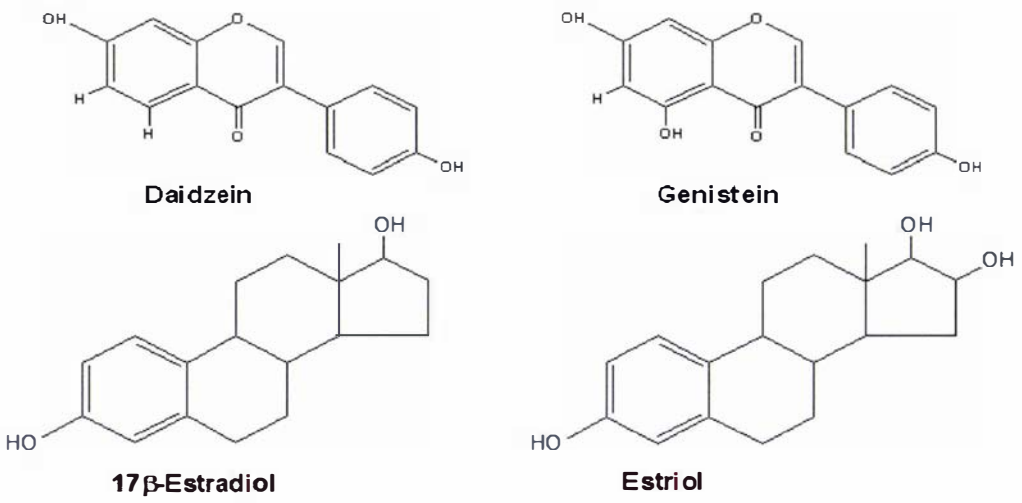

Figure 11 Structure of daidzein, genistein and two mammalian oestrogens

PHYTOESTROGEN METABOLISM

Genistein and daidzein are usually present in food as conjugated glycosides. In their glycoside-linked form, the two isoflavones are referred to as genistin and daidzin.

Unmetabolised genistein and daidzein are absorbed by passive transport from the small intestine [216, 217]. Genistein is catabolised by endogenous mammalian enzymes in the stomach and small intestine and also by enzymes of the colonic microflora. The metabolic pathway for genistein as proposed by Coldham *et al* in 2002 [218] is shown in **Figure 12**.

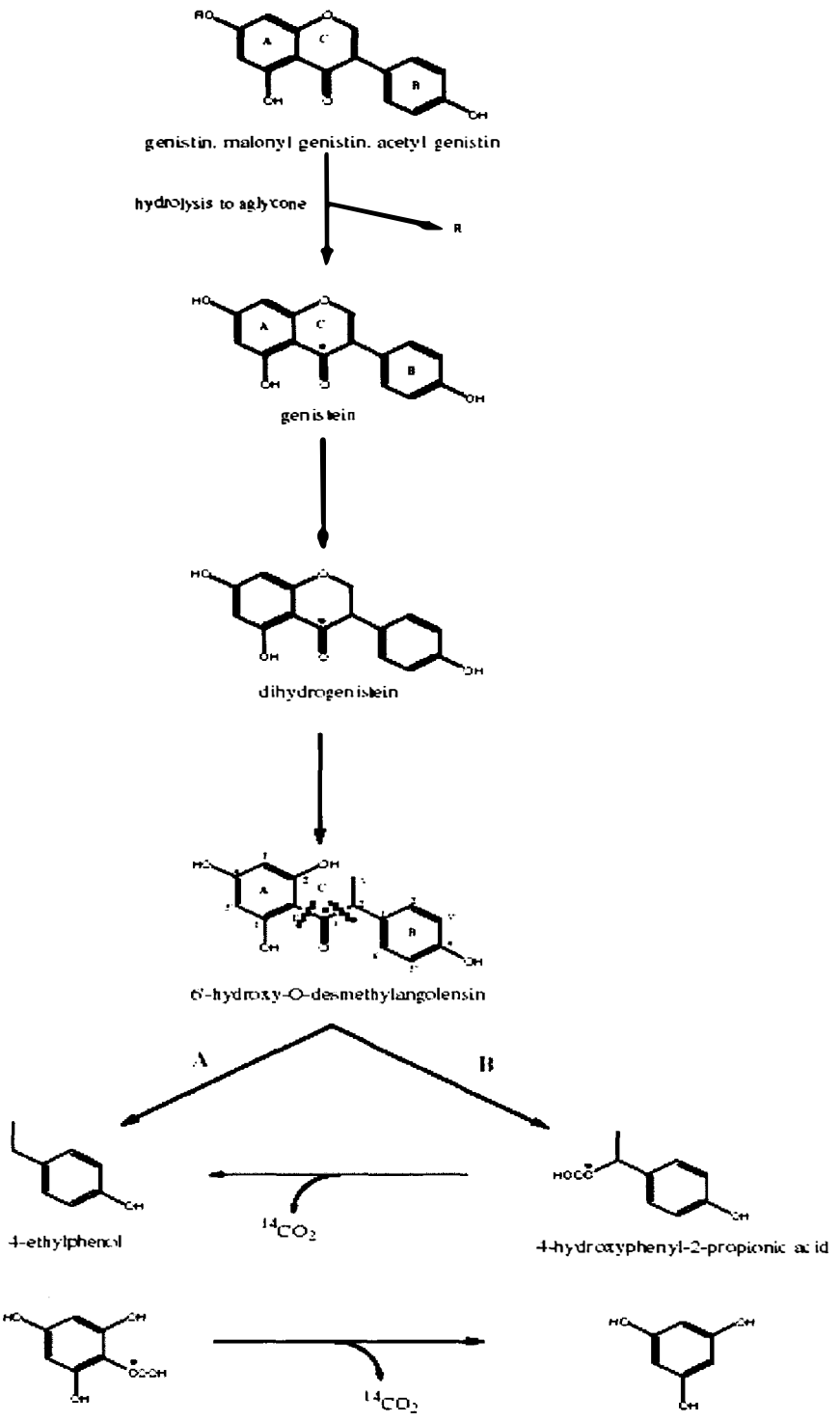

Figure 12 Metabolism of genistein. Reproduced from Coldham *et al*, 2002 [218].

In contrast to genistein, mammals appear to be incapable of catabolising daidzein. Microflora resident in the colon and distal small intestine catabolise daidzein to the two metabolites equol and o-desmethylangolensin (O-DMA) [219]. **Figure 13** shows the metabolic pathway for daidzein.

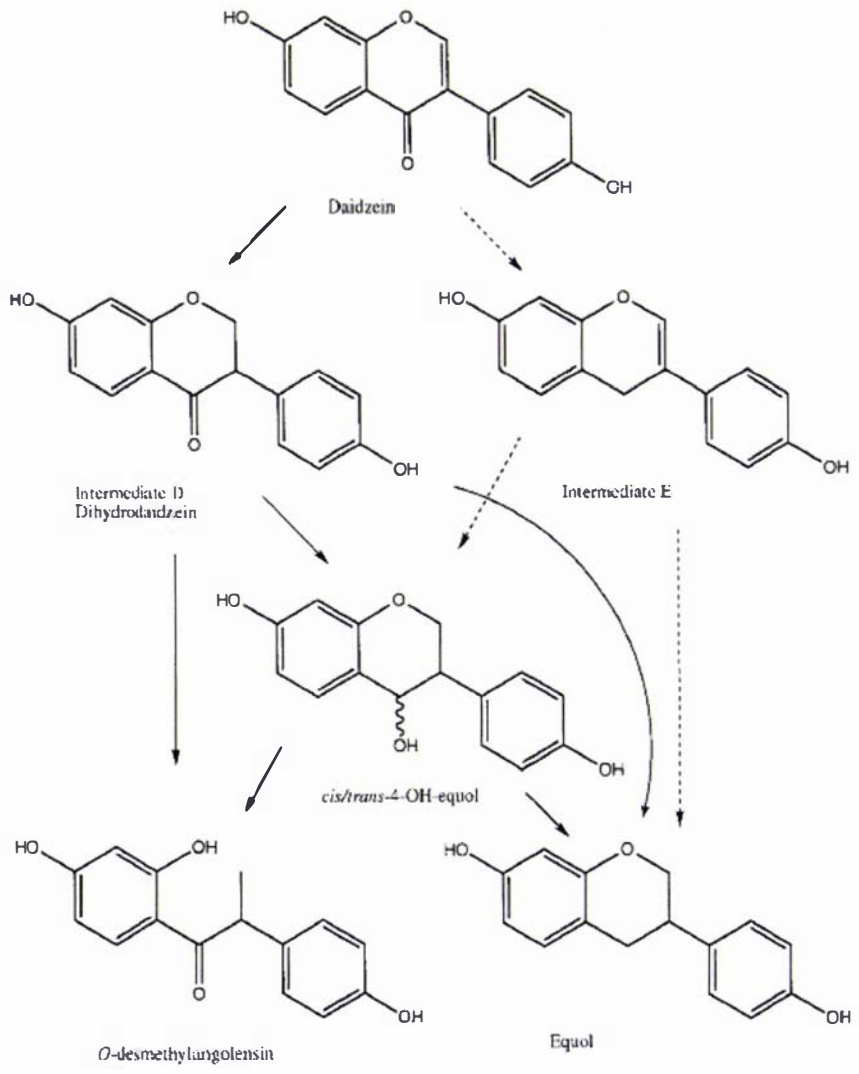

Figure 13 Metabolism of daidzein. Reproduced from Heinonen *et al*, 1999 [220].

Differences in isoflavone metabolism may exist between humans and other animals. For example rats may absorb isoflavone aglycones faster than glycosides [221] however the glycosidic conjugates appear to be more bioavailable than the aglycones in humans [215].

In Western societies where soy consumption is limited, approximately 33% of the population are equol producers [222]. On a global population basis, between 30% and

50% of all people may be capable of equol production. In contrast, all other mammals appear to produce equol [219, 223]. The lack of equol-producing ability in humans has been linked to the composition of the gut microflora. Germ-free rats colonised with gut microbes from equol-producing humans were capable of producing equol whereas those colonised with the gut microflora of a non-equol producing human were incapable of equol production [224]. Approximately 80-90% of humans are O-DMA producers [223]. O-DMA production in animals has not been well categorised however one study in non-human primates reported all were O-DMA producers [225].

Hereditary factors may contribute to isoflavone metabolising ability. Equol production appears to follow an autosomal dominant pattern of inheritance [223]. Racial differences in phytoestrogen metabolism may exist as O-DMA production has been reported to be less common in Asians than Caucasians [223] although the number of Asians, relative to Caucasians, tested in this study was small.

Genetics only accounts for part of the variation in isoflavone metabolism and environmental factors also play an important role [223]. O-DMA production in humans is inversely associated with age which may be due to changes in gut microflora over time [223]. There is some indication that increased isoflavone consumption promotes growth of flavonoid-metabolising bacteria in the colon [226]. It may therefore be possible for non-equol producing humans to acquire equol-producing ability. One trial in which the effect of a probiotic on equol-producing status was examined found equol production was significantly enhanced in 2 of the 40 subjects suggesting equol-producer status may be inducible in at least some individuals [227].

Other dietary components may also influence phytoestrogen metabolism. A high carbohydrate diet facilitates intestinal fermentation and as a result, more extensive biotransformation of phytoestrogens [228]. A positive relationship between total fat intake, total meat intake, the fat:fibre ratio of the diet and equol excretion was found in men and women consuming a traditional, high-soy, Japanese diet [229]. Short chain fatty acids, such as those derived from fructo-oligosaccharides by the action of colonic microbes, may enhance the bioavailability of isoflavones [230, 231].

Although one study has reported higher bioavailability of daidzein compared to genistein in women [232], this observation was probably a result of the differing clearance rates of genistein and daidzein rather any real difference in bioavailability. Other studies in which the half-lives of the two isoflavones in the blood has been accounted for have reported no difference in their relative bioavailability [233].

PHYTOESTROGENS AND BONE

In Asian populations, isoflavone intake is generally linked with higher BMD [234]. One large, epidemiological study found a positive association between total phytoestrogen intake and BMD in the lumbar spine and Ward's triangle in post-menopausal but not pre-menopausal Chinese women. Postmenopausal women in the highest tertile of phytoestrogen intake had significantly lower serum PTH, osteocalcin and urinary N-telopeptide excretion compared to those in the lowest tertile. When phytoestrogen intake was categorised into intakes of the three families of phytoestrogens namely flavonoids, coumesterol and lignans, no association was found between BMD and intakes of any of the phytoestrogen subfamilies [235].

In postmenopausal Japanese women, intake of soy protein [236] and genistein and daidzein [237] has been positively correlated with lumbar spine BMD. However urinary genistein, daidzein and equol excretion were not significantly correlated with lumbar spine BMD in healthy, osteopenic or osteoporotic, postmenopausal Korean [238].

Intervention Studies - Human

Whilst there is no evidence of an additive effect of daidzein and genistein on bone metabolism [239], it is known that different isoflavones have different biological effects. Soy protein itself may have beneficial effects on bone metabolism, independent of those of soy isoflavones [240, 241]. Although a considerable number of intervention studies have been carried out in both humans and animals, these studies have yielded mixed results. Interpretation of these studies is complicated in many cases by the omission of key information from published reports particularly in relation to disclosure of the composition and the amount of the isoflavone compound that was administered.

A summary of intervention studies examining the effects of soy isoflavones on bone in humans is provided in Appendix 1.

For the majority of human intervention studies which have been published in the scientific literature, the composition of individual isoflavones within the supplement used has not been divulged. It is known that different isoflavones have differing bioactivities. For instance genistein is a potent tyrosine kinase inhibitor whereas daidzein is not [242]. It therefore cannot be assumed that all isoflavones have the same effect on bone and hence it is important that the exact composition of isoflavones in the supplement used in an intervention trial is detailed.

A second major issue confounding the interpretation of the results of human intervention trials centres around determining the dose of isoflavone provided in the trial. As isoflavones are usually present in food as conjugated glycosides and glycosylation may influence isoflavone bioavailability [243], it is important that the form of isoflavone provided in intervention studies is reported. The glycosylation pattern of the isoflavone also influences the dose i.e. 20mg of genistin is not equivalent to 20mg of genistein. In order to be able to interpret and compare results of different studies, when a glycosylated isoflavone is used the dose should also be expressed in terms of aglycone equivalents.

The metabolism of isoflavones is complex. As there is considerable inter-individual variation in isoflavone metabolising-ability, the bioavailability of an isoflavone supplement within the study population needs to be assessed. Differences between study populations in terms of isoflavone-metabolising ability may impact considerably on trial outcome as in some instances an isoflavone metabolite may have greater bioactivity than the parent isoflavone. For example, equol has greater estrogenic and antioxidant activity than daidzein [219] and 4-ethylphenol, is more effective than genistein in modulating prostaglandin synthesis [244]. Few studies have sought to measure the bioavailability and extent of metabolite formation from the isoflavone supplement administered in the specific population studied.

Finally, many studies failed to adequately control for the confounding effects of other variables on bone mass, notably, dietary calcium intake. In several studies, calcium

intake was not reported [245-250]. However in one of these trials the isoflavone source was soy milk [249] and in another, both tofu and soy milk were among the selection of foods participants in the isoflavone intervention group could choose to include in their diet [248]. Soy milk and tofu are good sources of calcium and soy milk is often fortified with vitamin D therefore dietary calcium and/or vitamin D intake could also have been expected to increase in these studies again confounding the effects of the isoflavones. In several studies calcium intake was substantially altered during the trial period [241, 251, 252]. The confounding effect of changing dietary calcium was not controlled for in these studies therefore the changes in biochemical markers or BMC/BMD reported may largely have resulted from changing calcium rather than isoflavone intake.

One well-controlled intervention study reported isoflavone tablets providing 26mg biochanin A, 16mg formonectin, 1mg genistein and 0.5mg daidzein per day, ameliorated the decrease in lumbar spine (but not hip) BMC and BMD apparent in non-supplemented controls over a 12 month period. Subjects included pre-, peri- and post-menopausal women however the vast majority were post menopausal. Dietary calcium intake was relatively high at approximately 1000mg/day and vitamin D intake was approximately 3μg/day. Although the extent of metabolite formation was not assessed, urinary excretion of total isoflavones was measured in this study [253] therefore some measure of bioavailability could be determined. In a similar study, 12-month supplementation of early post-menopausal women with genistein tablets (providing 54mg genistein/day) resulted in significant increases in femoral neck and lumbar spine BMD. The increase in BMD was comparable to that seen in a second HRT-treated study group. In addition, biochemical markers of bone resorption were significantly reduced and markers of bone formation significantly increased in the genistein-supplemented group. Habitual calcium intake in the study population was approximately 900mg/day [254]. Decreased bone resorption marker excretion following isoflavone supplementation has been reported in a number of other studies involving soy isoflavone supplementation [245, 246, 252, 254] but not necessarily with non-soy isoflavone supplementation [253]. Increased bone formation marker excretion is also common with isoflavone supplementation regardless of isoflavone source [253].

In general, isoflavone supplementation studies in pre-menopausal women tend to report either no effect on bone mass [255] or a possible negative effect in terms of increased

circulating concentrations of biochemical markers usually associated with bone resorption [256, 257]. In postmenopausal women, generally no or little effect of isoflavone supplementation is observed when dietary calcium intake exceeds 1200mg/day [251, 258, 259] but at lower levels of dietary calcium intake, isoflavones may aid in maintaining bone mass.

Intervention Studies – Animal

A number of studies have examined the effects of isoflavones on bone in both growing and mature animals. These are summarised in Appendix 2. As with LCPUFA supplementation, isoflavones appear to have different effects on bone modelling compared to bone remodelling. Studies in weanling rats have reported either no effect [260] or a slight reduction in bone density [261] with soy isoflavone supplementation. (Although the latter study involved isoflavone supplementation of rats for four generations therefore the reduction in bone density may be due to the effects of chronic exposure.) In older, non-ovariectomised rats, dietary isoflavone intake of ≥18mg aglycones/kg body weight/day has been associated with slight increases in BMD in the lumbar spine [262] however intakes of ≤10mg aglycones/kg body weight/day had no effect on BMD [262]. The composition of the isoflavone supplement used in this study was not disclosed. Increased tibial BMD and increased serum osteocalcin concentration has been reported in one study where 5mg genistein/kg body weight was administered by subcutaneous injection to 2 month old, non-ovariectomised rats [263]. It is not possible to determine whether the lower dose required to elicit an effect on bone in the latter study was a result of the different route of administration or a reflection of perhaps greater bioactivity of genistein compared to other isoflavones.

The dose required to have a beneficial effect on BMD in ovariectomised animals may be higher than that required in sham animals as subcutaneous injection of 2-month old ovariectomised rats with 5mg genistein/kg body weight for 21 days was associated with increased serum osteocalcin concentration but had no effect on tibial BMD [263]. Studies in ovariectomised animals suggest isoflavones may have a biphasic effect on bone. In ovariectomised mice, subcutaneous injection with 0.4mg isoflavones/day increased femoral BMD however a dose of 0.7mg/day had no effect on BMD [264].

As with human studies, in many cases the isoflavone composition and dose have been incompletely reported for intervention studies in animals. This confounds interpretation of the varying effects on bone observed following isoflavone supplementation. A variety of different animal models have also been used ranging from weanling intact rats to 12-month old ovariectomised rats. The dose required to elicit a bone-protective effect may differ in growing compared to skeletally mature animals. In growing, ovariectomised rats fed a low calcium diet, isoflavone doses as low as 4mg/kg body weight/day have been associated with beneficial effects on bone mass [265]. A higher dose (>20mg/kg body weight/day) may be required to elicit an effect in growing, [266] and skeletally mature (6-month old) ovariectomised rats [267] fed a calcium-adequate diet. Lower doses [268] and supplementation regimens which do not commence immediately following ovariectomy [269] may be ineffective. Finally in aged (12-month old) ovariectomised Wistar rats, doses as low as 10mg/kg body weight/day of daidzein or genistein have been associated with beneficial effects on BMD [270]. It is unclear whether genistein and daidzein are more potent than mixed isoflavone supplements or whether the aged rat is more sensitive to the effects of isoflavones on bone mass.

Mechanisms of Action

Phytoestrogens are best known for their ability to mimic oestrogen activity, however they can act as either oestrogen agonists or antagonists depending on biological conditions [215]. In addition, they have a number of other effects, independent of those of oestrogen.

Oestrogenic Effects

Phytoestrogens bind to both known subtypes of the oestrogen receptor (ERα and ERβ) however the isoflavones have a much greater affinity for ERβ than ERα [271]. The binding affinities of genistein and daidzein relative to 17β-estradiol for ERα are 0.7% and 0.2% respectively. In comparison, the binding affinities of genistein and daidzein for ERβ are approximately 13% and 1% of that of 17β-estradiol [242]. Phytoestrogens interact with the oestrogen receptors in a different manner to endogenous oestrogens. Whereas 17β-estradiol has a lipophylic region which is thought to influence receptor-binding, genistein and daidzein lack this region. Interaction of genistein rather than

mammalian oestrogens with ERβ leads to changes at a different position of the transactivation helix resulting in lower oestrogenic activity [271]. Phytoestrogens also stimulate transcription of ERα and ERβ [228]. Daidzein has been shown to selectively enhance nuclear ER-β levels. This is in contrast to 17β-estradiol which enhances expression of both ER-α and ER-β [272].

Anti-Oestrogenic Effects

Phytoestrogens act in a number of ways to oppose the action of mammalian oestrogens and other sex hormones. They stimulate the synthesis of Sex Hormone Binding Globulin (SHBG) resulting in an increase in the amount of protein-bound, and therefore unavailable, estrone and estradiol in the blood [228]. Phytoestrogens also inhibit several enzymes involved in the metabolism of sex hormones. These include: 5α-reductase (converts testosterone to dihydrotestosterone), 17β-hydroxysteroid dehydrogenase (regulates interconversion of testosterone and androstenedione as well as 17β-estradiol and estrone) and the human P450 aromatase system (involved in estrone metabolism) [228]. High dietary flavonoid, but not isoflavonoid, concentration inhibits aromatase [273]. Genistein, daidzein and equol compete with estradiol for Nuclear Type II Oestrogen-binding sites and as a result, regulate oestrogen-stimulated growth and proliferation [273].

Other Effects

Both genistein and daidzein act as weak antioxidants and are inhibitors of angiogenesis [228]. They are also known to inhibit thyroid peroxidase, a key enzyme involved in thyroid hormone synthesis [228].

Genistein inhibits tyrosine kinase activity and this may be one of the mechanisms by which it impedes cancer cell growth [228]. Other enzymes known to be inhibited by genistein include topoisomerases I and II and protein histidine kinase [273]. Genistein also inhibits leptin secretion by adipocytes which may impact on bone metabolism [274].

Effect on Calcium Balance

The effects of genistein and daidzein on intestinal calcium absorption appear to be minimal. No effect of soy protein, with or without isoflavones, was evident on calcium

absorption, excretion or overall calcium balance in white, US, postmenopausal women [275]. *In vitro*, genistein and daidzein reduced transepithelial calcium absorption in the human intestinal-like Caco-2 cell line when cells were grown in the presence of oestrogen but had no effect on calcium absorption in the absence of oestrogen [276].

Effect on Osteoblastogenesis and Osteoblast Activity

Both genistein and daidzein stimulate osteoblast proliferation, differentiation and activation by an ER-dependent mechanism [272, 277, 278]. Both isoflavones also promote bone nodule formation *in vitro*. Whilst the effects of genistein on bone nodule formation appear to be ER-dependent, daidzein may act in an ER-independent manner [279]. ER activation by genistein has been shown to increase NOS (nitric oxide synthase) activity and increase NO and cGMP formation [278].

Aside from ER activation, genistein [280] and daidzein [281] also activate PPARs. Daidzein dose-dependently activates PPAR-α, PPAR-δ and PPAR-γ [281] although whether it activates one or both subforms of PPAR- γ is unclear. PPAR activation can modulate ER activity and the balance between PPAR and ER activation may govern the balance between adipogenesis and osteoblastogenesis [281]. Daidzein has a biphasic effect on osteogenesis. At low concentrations it stimulates osteoblast differentiation however at high concentrations it promotes adipogenesis. Daidzein-induced activation of PPAR-γ increases with increasing daidzein dose however the effects of daidzein on PPAR-δ are biphasic and PPAR-δ activation is minimal at high daidzein concentrations. PPAR-δ activation is believed to be a major contributor to the mechanism by which daidzein promotes osteogenesis. In one i*n vitro* study, daidzein-mediated activation of PPAR-α had no effect on osteogenesis or adipogenesis but inhibited daidzein-induced ER-mediated transcriptional activity. Daidzein-mediated activation of PPAR-δ stimulated osteogenesis, up-regulated ER-mediated transcriptional activity but had no effect on adipogenesis whereas activation of PPAR-γ by daidzein inhibited osteogenesis and ER-mediated daidzein activity and stimulated adipogenesis [281].

Genistein also acts as a PPAR-γ ligand and high concentrations of genistein result in PPAR-γ-stimulated adipogenesis at the expense of osteogenesis [280]. Low concentrations of genistein promote osteogenesis [280] and in bone marrow stromal cells low concentrations of genistein decreased PPAR-γ protein expression during adipogenesis thereby inhibiting adipocyte formation. The mechanism appeared to be

ER-mediated and involve upregulation of TGF-β1 protein levels [282]. Similarly, genistein inhibited PPAR-γ activity in human osteoblasts through activation of ER-α [283]. The effect of genistein on PPAR-δ is unknown. Although genistein also activates PPAR-α [284], no direct effect of genistein-mediated PPAR-α activation on bone cells has been documented.

Treatment of MC3T3-E1 pre-osteoblast-like cells with genistein or daidzein inhibited IL-6 formation through ER activation as well as by an ER-independent mechanism [285-287]. Both genistein and daidzein inhibited the rise in PGE2 production resulting from exposure of MC3T3-E1 cells to TNF-α [287]. BMP-2 [288, 289] and Cbfa-1 [272, 278] protein synthesis are upregulated by daidzein and genistein.

Soybean isoflavones in conjunction with saponins (a family of plant-derived triterpenes and steroids conjugated with either alkaloids or glycosides) increased serum γ-carboxylated osteocalcin concentrations in healthy men and women [290]. γ-carboxylation of osteocalcin is usually performed by vitamin K and is essential for enabling hydroxyapatite binding in the bone matrix [290]. However whether the increase in serum γ-carboxylated osteocalcin concentration was due to the effects of isoflavones or of saponins is unknown.

Effect on Osteoclastogenesis and Osteoclast Activity

Both genistein and daidzein enhanced inducible nitric oxide synthase (iNOS) activity in RAW264.7 cells possibly by an ERα-mediated effect involving increased production of TNF-α, a cytokine known to stimulate iNOS expression [291]. Low dose genistein (10^{-8}M) decreased osteoclast number in bone marrow culture by decreasing osteoclast viability. Higher concentrations of genistein (10^{-5}M) attenuated osteoclast formation [292]. Serum concentrations of IL-1 and TNF-α (but not IL-6) were significantly lower in postmenopausal women consuming a soy-supplemented diet [293]. Similarly a significant reduction in serum IL-1β and TNF-α concentrations were observed in ovariectomised rats treated with genistein [294]. In RAW264.7 cells, RANKL reduces ERα expression (RAW264.7 cells do not express ERβ). Genistein, daidzein and 17β-estradiol stimulate ERα expression and promote proliferation but inhibit multi-nucleation of the RAW264.7 cells [295].

In osteoblasts isolated from trabecular bone from young piglets, daidzein increased secretion of both OPG and soluble RANKL (sRANKL) and increased concentration of membrane-bound RANKL by an ER-mediated mechanism [272]. However, the ratio of sRANKL:OPG in serum from post-menopausal women supplemented with genistein for 12 months was significantly lower than in non-supplemented controls [296] suggesting that genistein may have differing effects on OPG and sRANKL excretion in humans or when endogenous oestrogen concentrations are low. In the case of genistein, modulation of RANKL and OPG levels may be a result of inhibition of topoisimerase-II activity [297].

As well as altering osteoblastic expression of factors controlling osteoclastogenesis, daidzein has also been shown to promote apoptosis of osteoclast progenitors by an ER-mediated mechanism [298].

Genistein and daidzein but not genistin, also inhibit inward rectifier K+ channels in osteoclasts. This leads to membrane depolarisation, intracellular influx of Ca2+ and inhibition of osteoclast-mediated bone resorption [299]. Therefore phytoestrogens inhibit bone resorption by inhibiting osteoclast differentiation as well as activity.

A beneficial effect of combined supplementation with oestrogenic compounds and LCPUFAs on bone mass post-ovariectomy?

Supplementation of ovariectomised rats with a diester of GLA and EPA in conjunction with 17β-estradiol treatment resulted in higher femur calcium content than diester supplementation or 17β-estradiol treatment alone however the difference was not statistically significant [300]. This may indicate a slight additive or synergistic interaction between 17β-estradiol and LCPUFAs. Observations from this study gave rise to the hypothesis that LCPUFAs and phytoestrogens may have additive or synergistic effects when administered in tandem.

Watkins *et al*, (2005) observed that ovariectomised rats supplemented with a combination of menhaden oil (rich in n-3 LCPUFAs) and soy protein containing isoflavones had significantly lower serum concentrations of the bone resorption marker Pyd compared to rats supplemented with n-6 LCPUFAs in combination with isoflavone-

containing soy protein. However, serum Pyd concentration was not significantly different between rats supplemented with n-3 LCPUFAs and soy protein containing only trace levels of isoflavones and rats supplemented with n-3 LCPUFAs and soy protein containing higher levels of isoflavones suggesting the effect was due to the presence of soy protein rather than the soy isoflavones. Serum concentration of DPyd, another bone resorption marker, did not differ significantly between groups. There were no significant effects of combined soy/LCPUFA supplementation on tibial or femoral BMC and BMD or serum concentrations of bone formation markers [301]. Although this study concluded that in combination, soy isoflavones and n-3 LCPUFAs had a complementary effect on reducing ovariectomy-induced BMC loss, this conclusion is not supported by the results of the study. Menhaden oil contains a milieu of different LCPUFAs as well as other bone-active nutrients such as vitamin D. Similarly, the soy protein supplement used in this trial contained a variety of different isoflavones. As it seems likely that different LCPUFAs and different isoflavones have different bioactivities, it is possible that a specific LCPUFA-isoflavone combination may have had a more pronounced effect.

Another study which aimed to compare the effects of n-3 and n-6 LCPUFAs in conjunction with animal or vegetable protein on bone mass following ovariectomy, reported combined supplementation of n-3 LCPUFAs with soy protein had a slight beneficial effect on bone mass in the lumbar spine in mice. This was a factorial-design study in which both ovariectomised and sham-operated mice were fed diets supplemented with n-3 or n-6 LCPUFAs in conjunction with either animal or soy protein. At trial completion there was no significant difference in femoral BMD between ovariectomised and sham-operated mice receiving the n-3 LCPUFA/soy protein supplement. In contrast, in mice supplemented with n-6 LCPUFA and soy protein, femoral BMD was significantly lower in the ovariectomised group compared to shams receiving the same supplement as well as both sham and ovariectomised mice supplemented with n-3 LCPUFAs and either soy or animal protein [302]. However femoral BMD was lower in sham-operated mice receiving either the n-3 LCPUFA/soy protein supplement or the n-6 LCPUFA/soy protein supplement compared to sham-operated mice receiving either n-3 or n-6 LCPUFAs with animal protein. Femoral BMD in ovariectomised mice receiving the soy protein/n-3 LCPUFA supplement was not significantly different from ovariectomised mice receiving the animal protein/n-6

LCPUFA supplement but was significantly lower than in ovariectomised mice receiving the animal protein/n-3 LCPUFA supplement [302]. Therefore although this study concluded that combined supplementation of n-3 LCPUFAs with soy protein preserved bone mass post-ovariectomy, the results of this study demonstrate a protective effect of n-3 LCPUFAs on bone but only when the LCPUFAs are fed in combination with animal not soy protein. Neither the composition of the n-3 LCPUFA supplement nor the isoflavone content of the soy protein was reported in this study.

Finally, reduced bone turnover following ovariohysterectomy in both rats and dogs has been reported as a result of consumption of a supplement containing vitamin K1, vitamin D3, n-3 LCPUFAs and genistein [303]. However as all four of these compounds have anabolic effects on bone it is not possible to discern whether combined n-3 LCPUFA and genistein supplementation has a co-operative effect in maintaining bone mass in these two animal models of postmenopausal osteoporosis.

Although results from these studies indicate there may be an interaction between the activities of soy and LCPUFAs, whether this interaction is due to the effects of soy isoflavones or soy protein or both and whether it results in a positive or negative effect on bone mass remains to be determined.

Motivation and Objectives for the Thesis

LCPUFAs may form an integral part of the mechanism by which bone remodelling is normally regulated. The combination of aging, life-style choices and menopause may lead to a relative deficiency of very long-chain LCPUFAs, particularly those of the n-3 family. Increased intake of specific LCPUFAs could therefore be a means of circumventing some of the deleterious effects of oestrogen deficiency and aging on bone mass. There is a lack of knowledge regarding the relative effectiveness of individual LCPUFAs in preventing bone loss post-menopause or post-ovariectomy and of the mechanisms of action of LCPUFAs in bone.

Consumption of phytoestrogens with their intrinsic oestrogenic activity may partially compensate for the lack of endogenous oestrogen synthesis following menopause. In addition, the anti-inflammatory activity exhibited by some phytoestrogens may also aid in minimising bone loss. Whether combined treatment with phytoestrogens and LCPUFAs has greater therapeutic value than either treatment alone in preventing post-menopausal bone loss remains to be determined.

The major objectives of the present thesis were:

1. To determine the relative effectiveness of GLA, EPA and DHA on bone mass in the ovariectomised rat model (Chapter 2).
2. To provide further knowledge with regard to the mechanism of action of LCPUFAs in bone and in bone cells (Chapters 2, 3, 5-7).
3. To determine the effect of combined treatment with LCPUFAs and either a mammalian oestrogen or phytoestrogens on bone mass post-ovariectomy *in vivo,* and on osteoblasts *in vitro* (Chapters 4, 5 and 7).

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CHAPTER 2

Specific effects of gamma-linolenic, eicosapentaenoic and docosahexaenoic ethyl esters on bone post-ovariectomy in rats

The objective of the study presented in this chapter was to determine if different LCPUFAs have different effects on bone mass in the ovariectomised rat. The effect of combined treatment with a specific ratio of three LCPUFAs was also assessed. The ratio of LCPUFAs used for this combined supplement was primarily based on a combination purported to preserve bone mass in ovariectomised animals and for which a patent application has been lodged by Roche Vitamins (DSM).

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Abstract

Long chain polyunsaturated fatty acids (LCPUFAs) and their metabolites are involved in the regulation of bone metabolism. Increased dietary consumption of n-3 LCPUFAs, and possibly some n-6 LCPUFAs, may limit post-menopausal bone loss. The aim of this study was to determine the effects on bone of specific fatty acids within the n-3 and n-6 LCPUFA families in ovariectomised rats. Six-month old rats were ovariectomised or SHAM-operated and fed either a control diet ("OVX" and "SHAM") or a diet supplemented with 0.5g/kg body weight/day of gamma-linolenic ("GLA"), eicosapentaenoic ("EPA"), docosahexaenoic ("DHA") ethyl esters or a mixture of all three ("MIX") for 16 weeks. Bone mineral content (BMC), area (BA) and density (BMD) and plasma concentrations of IGF-1, vitamin D and parathyroid hormone (PTH) were determined. The ovariectomy-induced decrease in lumbar spine BMC was significantly attenuated by DHA. Periosteal and endosteal circumferences of the tibia were significantly greater in DHA and EPA compared to controls. Plasma IGF-1 concentration at trial completion tended to be higher in DHA than in SHAM, OVX and GLA. Plasma concentration of total 25-hydroxyvitamin D (D2 + D3) was higher, but plasma 25-hydroxyvitamin D3 concentration lower, in DHA compared to SHAM at the end of the supplementation period. Femur BMC decreased by a significantly greater amount over the duration of the study in GLA than in OVX. Plasma PTH concentrations at trial completion were significantly higher in GLA compared to all other groups. In conclusion, DHA ameliorated ovariectomy-induced bone mineral loss. The bone-preserving effect of DHA may have been due to elevation of IGF-1 concentrations and modulation of vitamin D metabolism. GLA exacerbated post-ovariectomy bone mineral loss possibly as a result of PTH-induced bone catabolism.

Introduction

Although best known for their cardio-protective role, long-chain polyunsaturated fatty acids (LCPUFAs) and their metabolites also regulate bone metabolism and consequently, may have potential in the prevention and/or treatment of osteoporosis [1-3]. The integral role of prostaglandin E2 (PGE2), a cyclooxygenase metabolite of the n-6 LCPUFA arachidonic acid (AA 20:4n-6), in the regulation of osteoblast and osteoclast formation and function is well recognised. However a range of other LCPUFA metabolites, as well as various LCPUFAs themselves, are also bioactive and the

involvement of LCPUFAs in bone metabolism may be much more extensive than is currently recognised. For instance, resolvin E1 (RvE1), a newly-described lipoxygenase-generated lipid mediator derived from the n-3 LCPUFA eicosapentaenoic acid (EPA, 20:5n-3) inhibits osteoclastic bone resorption [4]. Similarly, lipoxin A4 (LXA4), a tri-hydroxylated lipoxygenase product of AA also inhibits osteoclast activity [5] whereas mono-hydroxylated lipoxygenase products of AA such as leukotriene B4 and 5-hydroxyeicosatetraenoic acid (5-HETE) inhibit bone formation [6].

Dietary manipulation of LCPUFA intake can influence bone metabolism and bone mass during bone growth [7-12] as well as at maturity [8, 9]. Whereas a negative association between total dietary LCPUFA intake and bone mass has been reported in one longitudinal study in post-menopausal Western women [13], both epidemiological and longitudinal studies have reported a positive relationship between intake of the n-3 family of LCPUFAs and bone mineral density [1, 14] suggesting the n-3 LCPUFA family may have bone-protective effects.

Intervention studies in animals supplemented with n-3 LCPUFAs report increased calcium balance and bone formation rate during growth [15] as well as preservation of bone mass post-ovariectomy in mature animals [8]. Combined supplementation of n-3 LCPUFAs with the n-6 LCPUFA, gamma-linolenic acid (GLA, 18:3n-6) has also been linked with anabolic effects on bone, post-menopause in women [9] and post-ovariectomy in rodents [16]. Few studies have investigated the effects of specific LCPUFAs on bone mass.

It is unclear if all LCPUFAs are bone-active and if so, if they act by the same or different mechanisms to regulate bone metabolism. The aim of the present study was to determine the effects of GLA and the two n-3 LCPUFAs eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) on bone in the ovariectomised rat, a model used for study of postmenopausal bone loss [18].

Methods

Animals

Sixty-eight female Sprague-Dawley rats aged 7 months, were randomly assigned to one of six groups and either ovariectomised (OVX) (1 group, n=10; 4 groups, n=12) or sham operated (1 group, n=10) under general anaesthetic (isofluorane). Sham operated animals were anaesthetised and an incision made in the peritoneal wall but the ovaries left intact.

Animals were housed individually in shoebox cages at 22 (± 2) °C with a 12h light/dark cycle in a dedicated room. The study was approved by the Massey University Animal Ethics Committee (Approval number 03/102).

Diets

Animals were given a nutritionally balanced, semi-synthetic diet (comprising 14% casein, 5% cellulose, 4% corn oil, 0.5% calcium, 59.7% starch, 6% sucrose with added vitamins and minerals as shown in **Table 1**) for four weeks prior to ovariectomy (week -4 to week 0). The diet formulation was based on AIN93M [19] with added vitamins and minerals as necessary to compensate for the nutrient content of local ingredients [20]. The type of oil in the diet was also altered from soybean (as stipulated for AIN93M) to corn oil as soybean oil is a source of n-3 LCPUFAs. The sham-operated ("SHAM", n=10) and ovariectomised control ("OVX", n=10) groups were maintained on this diet for the entire 16 week study period. Immediately following ovariectomy, the experimental groups (n=12 per group) were fed diets in which some of the corn oil was replaced with ethyl esters of either EPA (90% purity, 01177A-E90 Sanmark LLC, USA), GLA (95% purity, Crossential GLA E95 Croda Chemicals Ltd, UK), DHA (80% purity, 01177B-E80 Sanmark LLC, USA) or a mixture of EPA, GLA and DHA (ratio 1:2:4) at a dose of 0.5g/kg body weight/day. All diets contained 4% total fat and at least 2% corn oil, an amount in excess of the minimum amount required to prevent n-6 LCPUFA deficiency (1%). Diets were randomly sampled and analysed chemically to confirm the formulated nutrient contents. Diet compositions are given in **Table 1**.

Table 1 *Ingredient composition (% air-dry weight) of control and experimental diets.*

	Treatment Group					
	SHAM	**OVX**	**GLA**	**EPA**	**DHA**	**MIX**
Cornstarch	59.7	59.7	59.7	59.7	59.7	59.7
Sodium Caseinate	14	14	14	14	14	14
Sucrose	6	6	6	6	6	6
Cellulose	5	5	5	5	5	5
Vitamins[a]	5	5	5	5	5	5
Minerals (excl. Ca)[b]	5	5	5	5	5	5
Calcium Carbonate	1.3	1.3	1.3	1.3	1.3	1.3
Corn Oil[c,d]	4	4	3.3 – 3.1	3.3 – 3.1	3.3 – 3.1	3.3 – 3.1
GLA ethyl ester[c]	0	0	0.7 – 0.9	0	0	0.2 – 0.3
EPA ethyl ester[c]	0	0	0	0.7 – 0.9	0	0.1
DHA ethyl ester[c]	0	0	0	0	0.7 – 0.9	0.4 -0.5

[a] Supplying (mg/kg diet) retinol acetate 5.0, $_{DL}$-α-tocopherol acetate 200.0, menadione 3.0, thiamine hydrochloride 5.0, riboflavin 7.0, pyridoxine hydrochloride 8.0, D-pantothenic acid 20.0, folic acid 2.0, nicotinic acid 20.0, D-biotin 1.0, myo-inositol 200.0, choline chloride 1500; (μg/kg diet) ergocalciferol 25.0, cyanocobalamin 50.0.

[b] Supplying (g/kg diet) chloride 7.79, magnesium 1.06, phosphate 4.86, potassium 5.24, sodium 1.97; (mg/kg diet) chromium 1.97, copper 10.7, iron 424, manganese 78.0, zinc 48.2; (μg/kg diet) cobalt 29.0, iodine 105.0, molybdenum 152.0, Selenium 151.0.

[c] LCPUFA dose was 0.5g/kg rat body weight/day. Percentage of LCPUFA in diets increased and percentage of corn oil decreased as the body weight of animals increased over the trial period. Values given are the minimum and maximum dietary proportions of corn oil or LCPUFA provided at trial commencement and trial completion.

[d] Fatty acid composition of corn oil: 58% linoleic acid, 28% oleic acid, 11% palmitic acid and 2% stearic acid.

GLA = Gamma-linolenic acid (18:3n-6)
EPA = Eicosapentaenoic acid (20:5n-3)
DHA = Docosahexaenoic acid (22:6n-3)

The LCPUFAs and corn oil were blended into the experimental diet on a daily basis to prevent PUFA oxidation. Body weights of all animals were measured weekly, and the amount of LCPUFA and corn oil added to each test diet was adjusted according to the mean body weight of animals in each treatment group. The SHAM group was fed *ad libitum*. The food intake of the ovariectomised animals was limited to that of SHAM to reduce ovariectomy-induced weight gain. All animals had *ad libitum* access to deionised water throughout the study period.

Dual Energy X-Ray Absorptiometry (DEXA) Scans

Femur (F) and lumbar spine (LS) (L1-L4) bone mineral content (BMC), area (BA) and density (BMD) were determined with a Hologic QDR4000 pencil beam bone densitometer (Bedford, USA). Prior to scanning, animals were anaesthetised with a mixture of Acepromazine (ACP), Ketamine, Xylazine and sterile water (2:5:1:2), at a

dosage of 0.05ml/100g body weight administered intra-peritoneally via a 25G x 5/8" needle. A suitable level of anaesthesia was attained after five to ten minutes and was maintained for up to 2 hours. Anaesthetised rats were placed in a supine, "frog-leg" position on an acrylic platform of uniform 1.5" thickness with femurs at right angles to the long axis of the spine and to the tibia. Regional high-resolution scans of both femurs and the lumbar spine were performed using a 0.06" diameter collimator with 0.012'' point resolution and 0.0254" line spacing. Scans were made at week –2 (baseline), week 5 and week 16 (endpoint). A quality control (QC) scan was undertaken daily to ensure precision met with the required coefficient of variation (CV). All scan analyses were performed by one operator. Repeatability of results was ascertained by scanning and analysing 2 femurs and 2 lumbar spines 10 times each. CVs for femur and lumbar spine bone areas were <1% and for both bone mineral content and density, <1.3%.

Blood Sampling

Two weeks prior to ovariectomy or sham operation (week -2), rats were placed in a purpose-built restrainer on top of a heat pad under a heat lamp. A single blood sample of approximately 1ml was withdrawn from the lateral tail vein, using a 23G x ¾" hypodermic needle and 1ml syringe. Blood samples were collected into vacutainers containing heparin, then centrifuged at 2000 rpm for 10 minutes. The plasma was removed, snap-frozen with liquid nitrogen, and then stored at –80 °C for later analysis of biochemical markers. Following the same procedure, a second blood sample was taken 8 weeks following ovariectomy and subsequently analysed for 17β-oestradiol (RIA, Diasorin, Saluggia, Italy) to confirm the success of ovariectomy.

Euthanasia and Dissection

At the end of the 16-week study period, animals were weighed, deeply anaesthetized via intra-peritoneal injection using a 25G x 5/8" needle and 1ml syringe with 0.1ml/kg body weight of a mixture consisting of acepromazine, ketamine, xylazine and sterile H_2O (2:5:1:2) and subsequently exsanguinated by cardiac puncture with a 19G x 1 ½" needle and 5ml syringe. The uteri and adnexae were removed and their wet weight determined as a second quality control measure to confirm success of ovariectomy. The lumbar spine and both rear legs were removed, and stored in phosphate buffered saline at -20°C pending pQCT, biomechanical testing and bone marrow fatty acid composition analysis.

Bone Marrow Fatty Acid Composition

Both epiphyses were removed from left femurs and bone marrow from the diaphysis was extracted into Kimax tubes under a stream of compressed air. Approximately 100mg of marrow was obtained from each femur. Bone marrow fatty acid composition was determined by direct transmethylation followed by gas chromatography. To each sample, 1ml of internal standard (2.5mg/ml tricosanoic acid methyl ester (C23:0, Sigma-Aldrich Chemicals) dissolved in chloroform), 2ml of toluene and 5ml of 5% sulphuric acid in methanol was added. Tubes were sealed, shaken and fatty acid methyl esters (FAMEs) formed by heating at 80°C for 1 hour. Samples were then cooled and shaken with 5ml of saturated NaCl, then centrifuged at 2000RCF for 10minutes at 10°C. The upper toluene layer was collected and 1µl with 1:100 split injected into an Agilent 6890 Gas Chromatograph with auto sampler and FID detector. A SGHE Sol Gel Wax column was used with a column length of 30 metres, internal diameter 0.25mm and film thickness 0.25µm. Hydrogen flow rate was 1.5ml/min, constant flow. Average linear velocity was 50cm/sec. The initial injection temperature was 170°C and temperature was ramped at 1°C/minute to 225°C. Fatty acids in the samples were determined by comparison with known standards supplied by Sigma-Aldrich Chemicals (37-component FAME mixture C4:0 – C24:0, PUFA 1 and PUFA 3) and Restek Bellefonte, PA, USA (NLEA FAME Mix, 28 components).

Biomechanical Testing

Right femurs were scraped free of adhering flesh and maintained in PBS at room temperature for 1 hour prior to testing. The length of each femur was measured with an electronic calliper and the midpoint marked. Both the anterior-posterior and latero-medial diameter at the midpoint of the femur were similarly determined.

The maximum load, breaking load, maximum deformity (stroke length), breaking stress, breaking strain, (the percent deformation of the femur just prior to the time of breaking) the breaking energy (the amount of energy required to break the femur) and elastic modulus (force required to bend the bone in the elastic phase of deformation) were determined using a Shimadzu Ezi-test (Shimadzu Corporation, Kyoto, Japan) materials testing machine. The femurs were subjected to a three point bending test with the application point of the upper fulcrum positioned midway between the two supporting

rods of the testing jig; the supporting rods were 15mm apart. Load was applied at a constant deformation rate of 50mm/min at the midpoint of the anterior surface of the femur.

Tibial Microarchitecture

Following completion of the trial, the opportunity arose to access a pQCT scanner. As both femurs had previously been subjected to destructive testing (biomechanical testing and bone marrow fatty acid analysis), the right tibia was used for pQCT analysis. After removal of skin and disarticulation, tibia length was determined manually with callipers, and the tibia was positioned for scanning on a plastic cradle. Scans were made with a XCT2000 pQCT scanner (Stratec, Pforzheim, Germany) 5mm from the proximal end of the tibia (at a constant site in the proximal metaphysis) and at 50% of the length of the tibia (mid-diaphysis). Voxel size was 0.1mm and scan speed was 5mm per second. Scans were analysed using the manufacturer's software; the contour threshold was 280mg/cm^3. Main outcome measures were trabecular BMC, BA and BMD (determined in the 5mm slice) and cortical BMC, BA and BMD, and endosteal and periosteal circumferences (determined at 50% of tibia length).

Blood Parameters

Plasma samples from baseline and endpoint were analysed for osteocalcin and c-terminal telopeptides of type 1 collagen (CTX) using Rat-MID Osteocalcin and "Ratlaps" ELISA kits (Osteometer Biotech, Herlev, Denmark).

At trial completion, plasma 25-hydroxyvitamin D3, total 25-hydroxyvitamin D2 + D3, 1,25-dihydroxyvitamin D2 + D3, intact parathyroid hormone (PTH) and insulin-like growth factor (IGF-1) concentrations were determined by immunoassay using commercially available kits as follows: 25-hydroxyvitamin D3 RIA, BioSource Europe SA, Nivelles, Belgium; Octeia 25-hydroxyvitamin D2 + D3 EIA, Immunodiagnostic Systems Ltd, Boldon, Tyne & Wear, UK; 1,25-dihydroxyvitamin D3 RIA, Immunodiagnostic Systems Ltd, Boldon, Tyne & Wear, UK; Rat BioActive Intact PTH ELISA Kits Immutopics Inc, San Clemente, California, USA (Cat # 60-2700) and IGF-1 RIA BioSource Europe SA, Nivelles Belgium.

Statistical Analysis

Bone densitometry measurements, as obtained by DEXA, were analysed for treatment effects by repeated measures mixed model analysis (PROC MIX) with post-hoc Tukey-Kramer testing using SAS 9.1®, SAS Institute, Carey, N.C., USA. In all cases the Toeplitz model was the best fit for the data. Plasma concentrations of CTX were log_{10}-transformed to avoid heteroscadascity. All other data conformed to the requirements of the general linear model. Comparisons between groups were made by one-way ANOVA with post-hoc Tukey testing using Minitab® 14, Minitab Inc., Pennsylvania, USA. A p-value of ≤ 0.05 was considered significant.

Results

Diets

The formulated fatty acid composition of the three LCPUFAs was confirmed by GC analysis (data not shown) and the proximate nutrient composition of the diets was confirmed by chemical analysis of randomly collected diet samples (data not shown).

Animals

There were no statistically significant between-group differences in body weight at baseline (mean weight 317.8 ± 3.1g). At the end of the trial period, there were no significant differences in mean body weight between ovariectomised groups regardless of dietary fatty acid treatment but all ovariectomised groups were significantly heavier than SHAM controls (mean body weight ovariectomised 387.0 ± 4.8g, mean weight SHAM 322.6 ± 12.0g). There were no significant differences among groups in the amount of food consumed (18-20g/day) Mean plasma concentration of 17β-oestradiol was 9.15 ± 0.65 pg/ml in SHAM but below the detectable limits of the assay in all ovariectomised animals, consistent with uterus weights at week 16 being significantly lower in ovariectomised rats compared to SHAM, indicating successful ovariectomy.

Bone Marrow Fatty Acid Composition

Dietary supplementation with each of the three LCPUFAs was reflected in bone marrow fatty acid composition (**Table 2**).

EPA and DPA represented a significantly higher proportion of total bone marrow fatty acids in the EPA group compared to all other groups. However, percentage of DHA in bone marrow fat was not significantly higher in the EPA group compared to OVX or GLA. Percentage of DHA in bone marrow was significantly higher in the DHA group compared to all other groups. Percentages of GLA and AA in bone marrow fat were significantly higher in the GLA group compared to all other groups. The percentages of EPA, GLA and DHA in bone marrow fat were higher in the MIX group compared to OVX and SHAM controls. The proportion of polyunsaturated fat in bone marrow was significantly higher, and the proportion of monounsaturated fat significantly lower in the GLA, EPA, MIX and DHA groups compared to SHAM and OVX. The proportion of saturated fat in bone marrow was significantly higher in all four treatment groups compared to OVX and in the EPA, DHA and MIX groups compared to SHAM. Although the relative proportions of the different n-3 fatty acids varied between the DHA and EPA groups, the ratio of total n-3 to total n-6 LCPUFAs in the EPA and DHA groups was identical and significantly higher than in all other groups.

Table 2 *Fatty acid composition (%) of bone marrow lipid*

	Treatment Group							
	SHAM	**OVX**	**GLA**	**EPA**	**DHA**	**MIX**	**SEM**	***p-value***
Linoleic acid	14.8^{a}	$14.1^{a,b}$	13.5^{b}	$14.2^{a,b}$	14.9^{a}	$14.2^{a,b}$	0.31	0.03
Gamma-linolenic acid	0.0^{a}	0.2^{b}	2.0^{c}	$0.1^{a,b}$	$0.1^{a,b}$	0.7^{d}	0.03	<0.001
Dihomogamma-linolenic acid	0.0^{a}	$0.1^{a,b}$	0.7^{c}	0.1^{b}	0.2^{b}	0.5^{c}	0.03	<0.001
Arachidonic acid	2.1^{a}	1.4^{b}	2.7^{c}	1.2^{b}	1.2^{b}	1.9^{a}	0.08	<0.001
Alpha-linolenic acid	0.2^{a}	0.5^{b}	0.4^{b}	0.4^{b}	0.4^{b}	0.4^{b}	0.03	0.03
Eicosapentaenoic acid	0.0^{a}	0.0^{a}	0.0^{a}	1.1^{b}	0.3^{c}	0.3^{c}	0.03	<0.001
Docosapentaenoic acid (n-3)	0.0^{a}	0.1^{a}	0.0^{a}	1.5^{b}	0.4^{c}	0.5^{c}	0.05	<0.001
Docosahexaenoic acid	0.1^{a}	$0.2^{a,b}$	$0.2^{a,b}$	0.5^{b}	2.5^{c}	1.7^{d}	0.08	<0.001
Total Saturated	$32.6^{a,b}$	31.6^{b}	$33.7^{a,c}$	34.5^{c}	34.9^{c}	34.8^{c}	0.48	<0.001
Total Monounsaturated	48.9^{a}	49.9^{a}	44.7^{b}	44.7^{b}	42.5^{b}	42.8^{b}	0.61	<0.001
n-3 Polyunsaturated	0.3^{a}	0.7^{a}	0.6^{a}	$3.4^{b,c}$	3.6^{c}	3.4^{b}	0.15	<0.001
n-6 Polyunsaturated	$16.9^{a,b}$	15.7^{a}	18.9^{c}	15.6^{a}	$16.4^{a,d}$	$17.2^{b,d}$	0.33	<0.001
Total Polyunsaturated	17.2^{a}	16.4^{a}	19.4^{b}	19.0^{b}	20.0^{b}	20.2^{b}	0.34	<0.001
n-3 : n-6 ratio	0.02^{a}	0.04^{a}	0.03^{a}	0.22^{b}	0.22^{b}	0.17^{c}	0.01	<0.001

Fat was extracted from left femurs following ovariectomy or sham operation and after 16 weeks of dietary supplementation with gamma-linolenic acid (GLA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or a mixture of all three.
Different letters (a,b,c) denote significant differences among groups within the same row at $p \leq 0.05$.

Bone Mineral Content, Area and Density

At trial commencement there were no statistically significant differences between groups in lumbar spine BMC, BA and BMD (**Table 3**). On one-way ANOVA, a statistically significant difference between groups for baseline F BMC (p=0.01) and F BA (p=0.04) was apparent although no significant differences between any two groups were evident upon post-hoc testing (**Table 5**). For this reason, baseline BMC, BA and BMD for both LS and F were used as covariates in the statistical analysis.

Between-Group Comparisons of Change in BMC, BA and BMD

Ovariectomy resulted in a decrease from baseline in LS BMC of 18.1% over the study period (**Table 4**). Neither of the EPA, MIX or GLA treatments prevented the ovariectomy-induced decline in LS BMC. In contrast, LS BMC decreased by only 6.3% in the DHA group over the treatment period. Final LS BMC in the DHA group was significantly higher than in the OVX group ($p=0.03$) and was not significantly different from that of the Sham group ($p=0.77$) **(Table 3).** Although the net change in LS BA over the 16-week period in the OVX control group was very small (-0.8%) (**Table 4**), LS BA increased in both the DHA group (+7.1%, $p=0.0002$ compared to OVX), and to a lesser extent the MIX group (+5.1%, $p=0.07$ compared to OVX), over the treatment period. Final LS BA in both the DHA and MIX groups was significantly greater than OVX ($p=0.002$ and $p=0.04$ respectively). LS BMD decreased in both LCPUFA-treated and untreated ovariectomised rats over the study period ($p<0.0001$ compared to SHAM). Final LS BMD was not statistically significantly different among the ovariectomised groups **(Table 3)**.

F BMC decreased by 5.6% in OVX controls over the study period (**Table 4**). The decrease in F BMC in the GLA group was significantly greater than in OVX controls ($p=0.05$). Final F BMC in the GLA group was lower than in the DHA group although this failed to reach statistical significance ($p=0.07$). Final F BMC in the DHA group was not significantly different from SHAM ($p=0.27$) or OVX ($p=1.0$). At trial completion, F BA was significantly higher in EPA and DHA compared to sham ($p=0.0002$ and $p=0.0085$ respectively) **(Table 5)**.

Table 3 *Lumbar spine bone mineral content, area and density*

	Lumbar Spine Bone Mineral Content				***Within group difference p-values***		
	Baseline	Wk 5	Wk 16 (Final)	*SE*	***Wk 5 vs Baseline***	***Wk 16 vs Wk 5***	***Wk 16 vs Baseline***
Sham	0.50^{a}	0.49^{a}	0.50^{a}	*0.02*	*0.45*	*0.22*	*0.97*
OVX	0.47^{a}	0.43^{a}	0.39^{b}	*0.02*	*<0.0001*	*<0.0001*	*<0.0001*
GLA	0.50^{a}	0.47^{a}	$0.41^{b,c}$	*0.02*	*0.003*	*0.0013*	*0.0005*
EPA	0.47^{a}	0.45^{a}	$0.42^{b,c}$	*0.02*	*0.15*	*0.0002*	*0.12*
DHA	0.49^{a}	0.48^{a}	$0.45^{a,c}$	*0.02*	*0.36*	*0.23*	*0.50*
MIX	0.48^{a}	0.43^{a}	$0.43^{b,c}$	*0.02*	*<0.0001*	*0.0004*	*<0.0001*
Between group difference p-values	***0.64***	***0.06***	***<0.0001***		***Repeated Measures ANOVA***		
					Time		*p<0.0001*
					Treatment		*p=0.0007*
					Time × Treatment		*p<0.0001*
	Lumbar Spine Bone Area				***Within group difference p-values***		
	Baseline	Wk 5	Wk 16 (Final)	*SE*	***Wk 5 vs Baseline***	***Wk 16 vs Wk 5***	***Wk 16 vs Baseline***
Sham	1.99^{a}	1.99^{a}	$2.02^{a,b}$	*0.04*	*0.97*	*0.94*	*0.97*
OVX	1.94^{a}	1.96^{a}	1.95^{a}	*0.04*	*0.29*	*0.16*	*1.00*
GLA	2.01^{a}	2.06^{a}	$2.03^{a,b}$	*0.04*	*0.009*	*0.56*	*0.96*
EPA	1.95^{a}	2.02^{a}	$2.03^{a,b}$	*0.04*	*0.058*	*0.81*	*0.04*
DHA	1.97^{a}	2.07^{a}	2.10^{b}	*0.04*	*<0.0001*	*0.01*	*<0.0001*
MIX	1.94^{a}	1.96^{a}	2.06^{b}	*0.04*	*0.37*	*0.001*	*<0.0001*
Between group difference p-values	***0.47***	***0.04***	***<0.0001***		***Repeated Measures ANOVA***		
					Time		*p=0.004*
					Treatment		*p=0.10*
					Time × Treatment		*p=0.02*
	Lumbar Spine Bone Mineral Density				***Within group difference p-values***		
	Baseline	Wk 5	Wk 16 (Final)	*SE*	***Wk 5 vs Baseline***	***Wk 16 vs Wk 5***	***Wk 16 vs Baseline***
Sham	0.25^{a}	0.24^{a}	0.24^{a}	0.003	1.0	1.0	1.0
OVX	0.24^{a}	0.22^{b}	0.20^{b}	0.003	<0.0001	0.19	<0.0001
GLA	0.25^{a}	0.22^{b}	0.20^{b}	0.003	<0.0001	<0.0001	<0.0001
EPA	0.24^{a}	0.22^{b}	0.20^{b}	0.003	0.0001	0.006	<0.0001
DHA	0.25^{a}	$0.23^{a,b}$	0.21^{b}	0.003	<0.0001	0.01	<0.0001
MIX	0.25^{a}	0.22^{b}	0.21^{b}	0.003	<0.0001	<0.0001	<0.0001
Between group difference p-values	***0.87***	***0.0003***	***<0.0001***		***Repeated Measures ANOVA***		
					Time		*p<0.0001*
					Treatment		*p<0.0001*
					Time × Treatment		*p<0.0001*

Measurements were made in vivo by DEXA at baseline and 5 and 16 weeks following ovariectomy or sham operation and 16 weeks of dietary supplementation with gamma-linolenic acid (GLA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or a mixture of all three. Results are expressed as Least square with SE in ( ). Different letters (a,b,c) denote significant differences among groups within the same column at $p \leq 0.05$.

Table 4 *Percentage change in lumbar spine and femur bone mineral content, area and density*

	Treatment Group							
	SHAM	**OVX**	**GLA**	**EPA**	**DHA**	**MIX**	**SE**	***p-value***
Lumbar Spine (Baseline-Week 5)								
BMC (g)	-0.9[a]	-9.3[a]	-6.4[a]	-5.0[a]	-2.2[a]	-9.8[a]	1.19	*0.09*
BA (cm^2)	0.3[a]	1.3[a]	2.9[a]	3.6[a]	5.0[a]	1.4[a]	2.37	*0.08*
BMD (g/cm^2)	-0.4[a]	-2.5[b]	-2.3[b]	-2.0[b]	-1.7[a,b]	-2.7[b]	0.32	*0.0002*
Lumbar Spine (Week 5-Week 16)								
BMC (g)	4.7[a]	-8.4[b]	-8.9[b]	-8.7[b]	-4.0[a,b]	-2.1[a,b]	*2.49*	*0.005*
BA (cm^2)	2.8[a,b]	-1.3[a]	0.4[a,b]	0.3[a,b]	2.1[a,b]	4.0[b]	*1.27*	*0.05*
BMD (g/cm^2)	1.9[a]	-7.4[b]	-9.5[b]	-9.0[b]	-6.1[b]	-5.9[b]	*1.57*	*0.0002*
Lumbar Spine (Baseline-Week 16)								
BMC (g)	2.5[a]	-18.1	-15.1[ab]	-13.2[b,c]	-6.3[c]	-11.8[b,c]	*2.63*	*<0.0001*
BA (cm^2)	3.1[a,b]	-0.8[a]	3.2[a,b]	3.8[a,b]	7.1[b]	5.1[b]	*1.39*	*0.01*
BMD (g/cm^2)	-0.7[a]	-18.1[b]	-17.9[b]	-16.7[b]	-12.6[b]	-16.3[b]	*1.59*	*<0.0001*
Femur (Baseline-Week 5)								
BMC (g)	-0.8[a]	-7.3[a,b]	-8.0[b]	-8.2[b]	-5.9[a,b]	-8.1	*1.70*	*0.04*
BA (cm^2)	0.4[a]	0.8[a]	3.4[a]	4.2[a]	3.7[a]	2.5[a]	*1.08*	*0.06*
BMD (g/cm^2)	-1.6[a]	-6.2[a,b]	-11.0[b]	-11.8[b]	-8.7[b]	-10.1[b]	*1.67*	*0.0002*
Femur (Week 5-Week 16)								
BMC (g)	5.2[a]	1.9[a,b]	-1.9[b]	1.2[a,b]	0.8[a,b]	0.8[a,b]	*1.19*	*0.005*
BA (cm^2)	2.7[a]	5.1[a]	2.3[a]	3.4[a]	4.8[a]	3.7[a]	*0.89*	*0.14*
BMD (g/cm^2)	2.5[a]	-3.0[b]	-4.1[b]	-2.0[b]	-3.9[b]	-2.8[b]	*0.80*	*<0.0001*
Femur (Baseline-Week 16)								
BMC (g)	4.1[a]	-5.6[b]	-9.8[b]	-7.3[b]	-4.6[b]	-6.7[b]	*1.65*	*<0.0001*
BA (cm^2)	2.9[a]	5.9[a,b]	5.7[a,b]	7.4[b]	8.5[b]	6.5[b]	*0.99*	*0.001*
BMD (g/cm^2)	0.9[a]	-8.3[b]	-14.6[c]	-13.7[b,c]	-12.3[b,c]	-12.5[b,c]	*1.49*	*<0.0001*

Bone mineral content (BMC), bone mineral density (BMD) and bone area (BA) were measured by DEXA at baseline and weeks 5 and 16 following ovariectomy or sham operation and dietary supplementation with gamma-linolenic acid (GLA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or a mixture of all three. Results are expressed as Least square with SE in ( ). Different letters (a,b,c) denote significant differences among groups within the same row at $p \leq 0.05$.

Table 5 *Femur bone mineral content, area and density..*

	Femur Bone Mineral Content				***Within group difference p-values***		
	Baseline	Wk 5	Wk 16 (Final)	*SE*	***Wk 5 vs Baseline***	***Wk 16 vs Wk 5***	***Wk 16 vs Baseline***
Sham	0.53[a]	0.52[a]	0.53[a]	*0.01*	*0.81*	*0.30*	*0.79*
OVX	0.49[a]	0.46[b]	0.48[b]	*0.01*	*0.0008*	*0.47*	*0.02*
GLA	0.53[a]	0.49[b]	0.46[b]	*0.01*	*<0.0001*	*0.057*	<0.0001
EPA	0.50[a]	0.46[b]	0.48[b]	*0.01*	*<0.0001*	*0.91*	*0.002*
DHA	0.53[a]	0.50[a,b]	0.49[a,b]	*0.01*	*0.009*	*0.19*	*0.004*
MIX	0.50[a]	0.46[b]	0.48[b]	*0.01*	*<0.0001*	*0.85*	*0.0002*
Between group difference p-values	***0.01***	***0.002***	***<0.0001***		***Repeated Measures ANOVA***		
					Time		*p=0.0002*
					Treatment		*p=0.01*
					Time × Treatment		*p=0.04*
	Femur Bone Area				***Within group difference p-values***		
	Baseline	Wk 5	Wk 16 (Final)	*SE*	***Wk 5 vs Baseline***	***Wk 16 vs Wk 5***	***Wk 16 vs Baseline***
Sham	1.58[a]	1.59[a]	1.63[a]	*0.02*	*0.87*	*0.13*	*0.10*
OVX	1.58[a]	1.59[a,b]	1.67[a,b]	*0.02*	*0.69*	*0.001*	*0.002*
GLA	1.62[a]	1.66[b]	1.67[a,b]	*0.02*	*<0.0001*	*<0.0001*	<0.0001
EPA	1.56[a]	1.63[a,b]	1.70[b]	*0.02*	*0.005*	*<0.0001*	<0.0001
DHA	1.61[a]	1.66[b]	1.72[b]	*0.02*	*0.0003*	*<0.0001*	<0.0001
MIX	1.57[a]	1.60[a,b]	1.68[a,b]	*0.02*	*0.02*	*<0.0001*	<0.0001
Between group difference p-values	***0.04***	***0.05***	***<0.0001***		***Repeated Measures ANOVA***		
					Time		*p=0.08*
					Treatment		*p<0.0001*
					Time ×Treatment		*p=0.49*
	Femur Bone Mineral Density				***Within group difference p-values***		
	Baseline	Wk 5	Wk 16 (Final)	*SE*	***Wk 5 vs Baseline***	***Wk 16 vs Wk 5***	***Wk 16 vs Baseline***
Sham	0.33[a]	0.33[a]	0.33[a]	0.003	0.88	0.01	1.0
OVX	0.32[a]	0.29[b]	0.29[b]	0.003	0.04	0.74	0.02
GLA	0.33[a]	0.29[b]	0.28[b]	0.003	<0.0001	0.009	<0.0001
EPA	0.32[a]	0.28[b]	0.28[b]	0.003	<0.0001	0.78	<0.0001
DHA	0.33[a]	0.30[b]	0.29[b]	0.003	<0.0001	<0.0001	<0.0001
MIX	0.32[a]	0.29[b]	0.28[b]	0.003	<0.0001	0.60	<0.0001
Between group difference p-values	***0.16***	***<0.0001***	***<0.0001***		***Repeated Measures ANOVA***		
					Time		*p<0.0001*
					Treatment		*p<0.0001*
					Time × Treatment		*p<0.0001*

Measurements were made in vivo by DEXA at baseline and 5 and 16 weeks following ovariectomy or sham operation 16 weeks of dietary supplementation with gamma-linolenic acid (GLA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or a mixture of all three. Results are expressed as Least square with SE in ( ). Different letters (a,b,c) denote significant differences among groups within the same column at $p \leq 0.05$.

Within-Group Pattern of Change with time in BMC and BA

Following repeated measures analysis, there were significant interactions between treatment and time for LSBMC ($p<0.0001$), LS BA ($p=0.02$), LS BMD ($p<0.0001$), F BMC ($p=0.04$), F BMD ($p<0.0001$) but not F BA. At week 5 post-surgery, LS BMC

was significantly lower in the OVX and MIX groups compared to baseline ($p<0.0001$) but LS BA was not significantly different from baseline in either group ($p=0.29$ OVX, $p=0.37$ MIX) (**Table 3**). In contrast, in the DHA and EPA groups, LS BMC at week 5 was not significantly different from baseline ($p=0.36$ and $p=0.15$ respectively). however LS BA at week 5 was higher than baseline ($p<0.0001$ DHA and $p=0.058$ EPA). In the GLA group, LS BMC was significantly lower, but LS BA was significantly higher at week 5 compared to baseline ($p=0.0034$ and $p=0.0092$ respectively, **Table 3**).

At week 16, LS BMC was significantly lower than at week 5 in OVX ($p<0.0001$), MIX ($p=0.0004$), EPA ($p=0.0002$) and GLA ($p=0.0013$) but not in SHAM ($p=0.22$) or DHA ($p=0.23$). LS BA at week 16 was not significantly different from week 5 in the EPA ($p=0.81$), GLA ($p=0.56$) and OVX ($p=0.16$) groups. However LS BA at week 16 was significantly greater than at week 5 in the DHA and MIX groups ($p=0.0097$ and $p=0.011$ respectively, **Table 3**).

F BMC at week 5 was significantly lower in all ovariectomised groups compared to baseline (DHA $p=0.0088$, OVX $p=0.0008$, EPA, GLA and MIX $p<0.0001$). F BA at week 5 was significantly higher than baseline in all LCPUFA-supplemented groups (DHA $p=0.0003$, EPA $p=0.0054$, GLA $p<0.0001$, MIX $p=0.024$,) but not in un-supplemented OVX ($p=0.69$) or SHAM controls ($p=0.87$). F BMC at week 16 tended to be lower than at week 5 in the GLA group ($p=0.057$), but was not significantly different from week 5 in any of the other groups (DHA $p=0.19$, EPA $p=0.91$, MIX $p=0.85$, OVX $p=0.47$, SHAM $p=0.30$). F BA at week 16 was significantly higher than at week 5 in all ovariectomised groups (DHA, EPA, MIX, GLA $p<0.0001$, OVX $p=0.0011$, **Table 5**).

Tibial Microarchitecture

At week 16, trabecular BMC in the OVX, EPA, MIX and GLA groups were lower than in SHAM controls ($p=0.01$, $p=0.0084$, $p=0.09$ and $p=0.02$ respectively) but there was no significant difference in trabecular BMC between SHAM and DHA ($p=0.19$). Cortical bone area was significantly greater in the DHA group compared to SHAM ($p=0.04$) and cortical BMC also tended to be greater in the DHA group compared to SHAM ($p=0.11$) **(Table 6)**. Periosteal circumference was significantly greater in the DHA and EPA groups compared to SHAM controls ($p=0.007$ and $p=0.02$ respectively). Endosteal circumference was also greater in these two groups compared to both OVX (DHA vs

OVX p=0.026 and EPA vs OVX, p=0.036) and SHAM (DHA vs SHAM p=0.034, EPA vs SHAM p=0.046) **(Table 6)**.

Table 6 *Trabecular and cortical bone mineral content (BMC), area (BA) and density (BMD) and periosteal and endosteal circumferences of right tibiae.*

	Treatment Group							
	SHAM	**OVX**	**GLA**	**EPA**	**DHA**	**MIX**	**SEM**	***p-value***
Trabecular BMC (mg/mm)	4.9^{a}	2.9^{b}	3.4^{b}	3.0^{b}	$3.8^{a,b}$	$3.6^{a,b}$	0.32	0.001
Trabecular BA (mm^2)	$9.5^{a,b}$	9.4^{a}	11.4^{b}	$10.1^{a,b}$	$11.1^{a,b}$	$10.7^{a,b}$	0.53	0.02
Trabecular BMD (mg/cm^3)	522.4^{a}	300.2^{b}	289.9^{b}	301.5^{b}	346.4^{b}	330.1^{b}	22.30	<0.001
Cortical BMC (mg/mm)	6.1^{a}	6.3^{a}	6.6^{a}	6.6^{a}	6.7^{a}	6.5^{a}	0.16	0.12
Cortical BA (mm^2)	4.6^{a}	$4.7^{a,b}$	$5.0^{a,b}$	$5.0^{a,b}$	5.1^{b}	$4.8^{a,b}$	0.11	0.03
Cortical BMD (mg/cm^3)	1332^{a}	1332^{a}	1325^{a}	1326^{a}	1319^{a}	1339^{a}	8.25	0.46
Periosteal circ. (mm)	9.6^{a}	$9.8^{a,b}$	$10.1^{a,b}$	10.2^{b}	10.3^{b}	$10.0^{a,b}$	0.13	0.004
Endosteal circ. (mm)	5.9^{a}	5.9^{a}	$6.1^{a,b}$	6.4^{b}	6.4^{b}	$6.2^{a,b}$	0.12	0.004

Measurements were made ex vivo by pQCT following ovariectomy or sham operation of female rats and after16 weeks of dietary supplementation with gamma-linolenic acid (GLA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or a mixture of all three. Trabecular BMC, BA and BMD were measured 5mm from the proximal end of the tibia. Cortical BMC, BA and BMD and periosteal and endosteal circumferences were determined at a slice taken 50% along the length of the tibia. Results are expressed as least square mean with SE in ( ). Different letters (a,b,c) denote significant differences among groups within the same row at $p \leq 0.05$.

Bone Biomechanics

As shown in **Table 7**, break stress (the maximum load per area of bone at breakpoint) tended to be lower in GLA compared to OVX (p=0.058) and SHAM (p=0.095). Femurs from GLA were significantly weaker in terms of the amount of bone deformation prior to breaking and energy absorbed prior to breaking, compared to femurs of EPA (p=0.046 and p=0.044 respectively). There were no significant differences among groups in terms of elastic modulus or break load.

Table 7 Biomechanical properties of right femurs

	Treatment Group							
	SHAM	**OVX**	**GLA**	**EPA**	**DHA**	**MIX**	**SEM**	***p-value***
Break load (N)	190.0^{a}	196.6^{a}	170.4^{a}	198.3^{a}	177.8^{a}	187.0^{a}	9.37	*0.23*
Max bone deformation (mm)	$1.7^{a,b}$	$1.7^{a,b}$	1.5^{a}	1.7^{b}	$1.6^{a,b}$	$1.6^{a,b}$	0.06	*0.04*
Break stress[#] (N/mm^2)	$85.5^{a,b}$	87.3^{a}	70.0^{b}	$79.4^{a,b}$	$74.8^{a,b}$	$78.7^{a,b}$	3.98	*0.05*
Elastic modulus (N/mm^2)	769.5^{a}	737.8^{a}	657.7^{a}	644.7^{a}	677.8^{a}	690.0^{a}	31.69	*0.08*
Energy absorbed prior to breaking (J)	$0.2^{a,b}$	$0.2^{a,b}$	0.1^{a}	0.2^{b}	$0.2^{a,b}$	$0.2^{a,b}$	0.01	*0.04*

Femurs were dissected from euthanased animals 16 weeks following ovariectomy or sham operation and after dietary supplementation with gamma-linolenic acid (GLA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or a mixture of all three. Biomechanical properties of the femur were determined by three-point bending. Results are expressed as least square mean with SE in ( ). Different letters (a,b,c) denote significant differences among groups within the same row at $p \leq 0.05$.

[#] Max load applied prior to breaking per area of bone at breakpoint.

Biochemical Markers of Bone Metabolism

There were no significant differences in plasma CTX concentration among study groups at baseline (data not shown). At trial completion however, plasma CTX concentration was greater in all ovariectomised groups compared to SHAM controls. The difference reached statistical significance compared to sham for the EPA, GLA and DHA groups **(Table 8)**.

There were no significant differences in plasma osteocalcin concentrations among groups at baseline (data not shown). However, at week 16 osteocalcin concentrations were significantly higher in EPA, DHA and MIX-supplemented ovariectomised groups compared to SHAM **(Table 8)**.

Table 8 *Biochemical marker, growth factor and hormone concentrations in plasma*

	Treatment Group							
	SHAM	**OVX**	**GLA**	**EPA**	**DHA**	**MIX**	**SEM**	***p-value***
Osteocalcin (ng/ml)	49.7^{a}	$87.9^{a,b}$	$96.0^{a,b}$	117.4^{b}	110.8^{b}	99.6^{b}	11.22	0.001
CTX (ng/ml)	4.9^{a}	$8.1^{a,b}$	9.7^{b}	12.3^{b}	9.0^{b}	$7.8^{a,b}$	1.15	<0.0001
IGF-1 (ng/ml)	943^{a}	952^{a}	960^{a}	1010^{a}	1065^{a}	984^{a}	30.3	0.07
25(OH) vitamin D2+D3 (nmol/l)	45.3^{a}	$55.0^{a,b}$	$60.9^{a,b}$	$67.8^{a,b}$	77.1^{b}	$51.0^{a,b}$	6.70	0.02
25(OH)vitamin D3 (nmol/l)	18.0^{a}	15.7^{a}	12.5^{a}	12.2^{a}	10.7^{a}	11.0^{a}	1.86	0.04
$1,25(OH)_2$ vitamin D2+D3 (pmol/l)	17.3^{a}	23.9^{a}	16.6^{a}	23.2^{a}	23.8^{a}	24.9^{a}	4.63	0.67
Bioactive intact PTH (pg/ml)	259^{a}	216^{a}	421^{b}	138^{a}	124^{a}	137^{a}	39.3	0.0001

Measurements were made by immunoassay following ovariectomy or sham operation and after 16 weeks of dietary supplementation with gamma-linolenic acid (GLA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) or a mixture of all three. Results are expressed as mean with SE in ( ). Different letters (a,b,c) denote significant differences among groups within the same row at $p \leq 0.05$.

IGF-1

Plasma IGF-1 concentration was higher in the DHA group compared to SHAM, OVX and GLA, however this difference failed to reach statistical significance (p=0.07 DHA vs SHAM and p=0.1 DHA vs GLA or OVX) **(Table 8)**.

Vitamin D and Parathyroid Hormone

Total 25-hydroxyvitamin D (D2 + D3) concentration was significantly higher in the DHA group compared to SHAM. Concentration of 25-hydroxyvitamin D3 was lower in DHA than in sham however the difference failed to reach statistical significance (p=0.06). Concentration of 25-hydroxyvitamin D3 in the MIX group also tended to be lower than sham (p=0.07). Total concentration of 25-hydroxyvitamin D2 and D3 was not significantly different in the MIX group compared to all other groups. There was no statistically significant difference among groups for plasma concentration of 1,25-dihydroxyvitamin D (D2 and D3) **(Table 8)**.

Plasma concentration of intact parathyroid hormone in the GLA group was significantly higher than in all other groups at week 16 **(Table 8)**.

Discussion

Dietary DHA supplementation resulted in amelioration of ovariectomy-induced bone mineral loss. A beneficial effect of DHA on bone mass post-ovariectomy has been previously suggested after studies comparing the effects of fish oil supplements with differing DHA contents [21, 22]. *In vitro*, DHA inhibits RANKL-induced differentiation of osteoclasts from RAW 264.7 cells [21]. DHA also inhibits transcription of cathepsin K, calcitonin receptor and MMP-9 and formation of resorption pits by RAW 264.7 cells [21] suggesting that at least part of the mechanism by which DHA preserves bone mass may be by inhibiting osteoclastogenesis. However the inhibitory effect of DHA on mature osteoclasts *in vivo* may be minimal or transient as no effect of DHA on bone resorption as measured by biochemical markers was observed by Kruger & Schollum (2005) in growing male rats [23]. Similarly, in the present study no effect on plasma concentration of the bone resorption marker CTX was observed in ovariectomised female rats. Rather, the greater endosteal circumference in tibiae from the DHA group in the present study implies a higher rate of bone resorption in the DHA group. Results from the present study suggest DHA may promote bone formation, particularly periosteal apposition of new bone. Whether this is a primary effect of DHA or a compensatory response to the increased endosteal resorption in this group remains to be determined. Upregulation of IGF-1 protein concentration due to either a change in rate of synthesis or turnover and modulation of vitamin D metabolism may contribute to the mechanism by which DHA acts in bone IGF-1 is known to stimulate periosteal bone apposition and trabecular remodelling [24] and to regulate vitamin D metabolism by modulating hydroxylase activity [25].

EPA supplementation had no discernible effect on BMC at either the lumbar spine or femur, but periosteal and endosteal circumferences were greater in the EPA group than OVX controls at trial completion. This suggests that although EPA treatment did not protect against ovariectomy-induced bone mineral loss, it may have influenced the site of bone remodelling. However, as endogenous enzymes can elongate and further desaturate EPA to form DHA albeit inefficiently, whether the observed endosteal and periosteal expansion is due to the effects of EPA or DHA cannot be ascertained. Low dose EPA consumption has previously been found to have no effect on bone mineral homeostasis in ovariectomised rats fed a calcium-adequate diet [26, 27] however higher

dose EPA (1.0g/kg body weight/day) supplementation has been shown to exacerbate bone mineral loss post-ovariectomy [26]. Although the effects of EPA on bone cells may be minimal, EPA may help to maintain calcium balance. Consumption of high EPA-containing fish oil has been associated with increased intestinal Ca2+-ATPase concentration [28], increased vitamin D receptor binding [28] and reduced faecal calcium excretion [10] in rats, suggesting EPA may promote intestinal calcium absorption. Sakaguchi *et al* (1994) reported preservation of bone calcium content in EPA-supplemented (160mg/day/kg diet) ovariectomised rats fed a calcium-deficient, but not a calcium-adequate diet [27]. Enhanced intestinal calcium absorption may have been the mechanism responsible for the bone mineral-preserving effect observed when dietary calcium intake was low. In the present study a calcium-adequate diet was fed and hence any beneficial effect of EPA in enhancing dietary calcium absorption is likely to have had minimal effect on bone.

GLA supplementation exacerbated the ovariectomy-induced decline in bone mineral content and density in the femur. Biomechanical testing also indicated femurs of the GLA supplemented group tended to be weaker than those of other groups. In the present study, the percentage of AA in bone marrow and the concentration of PTH in plasma were significantly higher in the GLA group compared to all other ovariectomised groups. GLA can be further metabolised by endogenous enzymes to DGLA and AA, substrates for cyclooxygenase-mediated synthesis of PGE1 and PGE2 respectively. Both PGE1 and PGE2 stimulate PTH expression and/or release resulting in an elevated plasma PTH concentration [29, 30]. Continual high PTH levels have a catabolic effect on bone [31, 32]. Therefore it is possible that the mechanism responsible for the reduction in F BMC and BMD observed in the GLA group was due to PTH and resulted from the actions of prostaglandin(s).

Interestingly, although EPA, DHA and (to a lesser extent) GLA treatments were associated with greater endosteal and periosteal circumferences in the tibia than in the SHAM group, periosteal and endosteal circumferences in the MIX group were not greater than in the OVX or SHAM group, This suggests that different, and perhaps opposing, mechanisms were employed by the three LCPUFAs to bring about this change.

In conclusion, GLA, DHA and possibly EPA are bioactive in bone *in vivo* but they have divergent effects and appear to act by different mechanisms. Under the study conditions, DHA was the most effective of the LCPUFAs tested at maintaining bone mineral content post-ovariectomy. Further work is required in order to clarify the mechanisms of action of DHA in bone.

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CHAPTER 3

Long chain polyunsaturated fatty acids alter membrane-bound RANKL expression and osteoprotegerin secretion by MC3T3-E1 osteoblast-like cells

Little is known about the mechanisms of action of long chain polyunsaturated fatty acids in bone. The aim of this study was to determine the effect of long chain polyunsaturated fatty acids on the RANKL/OPG signalling pathway, a well-characterised pathway governing osteoclast formation and activity.

Data published in: Poulsen RC, Wolber FM, Moughan PJ, Kruger MC Long chain polyunsaturated fatty acids alter membrane-bound RANKL expression and osteoprotegerin secretion by MC3T3-E1 osteoblast-like cells Prostaglandins and other Lipid Mediators.2007 In Press

Abstract

Inflammation triggers an increase in osteoclast (bone resorbing cell) number and activity. Osteoclastogenesis is largely controlled by a triad of proteins consisting of a receptor (RANK), a ligand (RANK-L) and a decoy receptor (osteoprotegerin, OPG). Whilst RANK is expressed by osteoclasts, RANK-L and OPG are expressed by osteoblasts. The long chain polyunsaturated fatty acid (LCPUFA) arachidonic acid (AA, 20:4n-6) and its metabolite prostaglandin E2 (PGE2), are pro-inflammatory and PGE2 is a potent stimulator of RANKL expression. Various LCPUFAs such as eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3) and gamma-linolenic acid (GLA, 18:3n-6) have anti-inflammatory activity. We aimed to determine if AA itself can stimulate RANKL expression and whether EPA, DHA and GLA inhibit RANKL expression in osteoblasts. MC3T3-E1/4 osteoblast-like cells were cultured under standard conditions with each of the LCPUFAs (5μg/ml) for 48 hours. Membrane-bound RANKL expression was measured by flow cytometry and OPG secretion measured by ELISA. In a second experiment, RANKL expression in MC3T3-E1/4 cells was stimulated by PGE2 treatment and the effect of EPA, DHA and GLA on membrane-bound RANKL expression and OPG secretion determined. The percentage of RANKL-positive cells was higher ($p<0.05$) than controls following treatment with AA or GLA but not after co-treatment with the cyclooxygenase inhibitor, indomethacin. DHA and EPA had no effect on membrane-bound RANKL expression under standard cell culture conditions. Secretion of OPG was lower ($p<0.05$) in AA -treated cells but not significantly different from controls in GLA, EPA or DHA treated cells. Treatment with prostaglandin E2 (PGE2) resulted in an increase ($p<0.05$) in the percentage of RANK-L positive cells and a decrease ($p<0.05$) in mean OPG secretion. The percentage of RANKL positive cells was significantly lower following co-treatment with PGE2 and either DHA or EPA compared to treatment with PGE2 alone. Mean OPG secretion remained lower than controls in cells treated with PGE2 regardless of co-treatment with EPA or DHA. Results from this study suggest COX products of GLA and AA induce membrane-bound RANKL expression in MC3T3-E1/4 cells. EPA and DHA have no effect on membrane-bound RANKL expression in cells cultured under standard conditions however both EPA and DHA inhibit the PGE2-induced increase in RANKL expression in MC3T3-E1/4 cells.

Introduction

Pro-inflammatory signalling has a fundamental role in the initiation of bone remodelling, triggering both osteoclastogenesis and osteoblastogenesis. Osteoclasts are large, multi-nucleated cells of the monocyte/macrophage lineage which resorb fatigued bone. Osteoblasts are mononuclear cells of mesenchymal origin which synthesise new bone tissue and replace that which osteoclasts remove. Under normal conditions the processes of bone resorption and formation are coupled and there is no net change in overall bone mass. However conditions leading to elevated levels of inflammatory mediators result in an imbalance between osteoclast and osteoblast formation and activity and consequently, an overall loss of bone mass [1].

Osteoclastogenesis is largely controlled by a triad of proteins consisting of two receptors, RANK (Receptor Activator of Nuclear factor κB) and OPG (osteoprotegerin), and a ligand, RANKL (Receptor Activator of Nuclear factor κB ligand) [2, 3]. RANK is a membrane-bound receptor present on osteoclast precursors [4]. RANKL and OPG are expressed by osteoblasts [5, 6] as well as various other cell types including activated T-cells [7] and fibroblasts [8]. OPG is a soluble protein whereas RANKL is largely expressed as a membrane-bound protein although small amounts of intact RANKL are also secreted by osteoblasts [9]. Binding of RANK to RANKL stimulates osteoclastogenesis and promotes mature osteoclast survival [10]. However binding of RANKL to OPG leads to a rapid decline in osteoclast number due to prevention of osteoclastogenesis, increased apoptosis of mature osteoclasts [10, 11], and a decrease in bone resorbing activity of existing osteoclasts [12]. Inflammatory mediators are involved in the regulation of RANKL and OPG expression [13]. The level of RANKL expression relative to OPG is a contributing factor to the regulation of osteoclastogenesis [14].

Prostaglandin E2 (PGE2), a pro-inflammatory lipid mediator derived from the n-6 long chain polyunsaturated fatty acid (LCPUFA) arachidonic acid (AA, 20:4n-6) by the activity of cyclooxygenase (COX), promotes osteoclastogenesis by stimulating RANKL expression and inhibiting OPG secretion by osteoblasts [15]. Similarly, treatment of osteoblasts with AA stimulates secretion of soluble RANKL and inhibits secretion of OPG, and both effects are largely blocked by COX inhibition [16].

The n-3 family of LCPUFAs, particularly eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) and another member of the n-6 LCPUFA family, gamma-linolenic acid (GLA, 18:3n-6), are generally regarded as anti-inflammatory lipids [17-19]. Dietary consumption of oils rich in EPA, DHA and/or GLA has been linked with anti-resorptive effects [20] or bone-protective effects [21, 22] in animal and human studies. The potential effects of these three LCPUFAs on membrane-bound RANKL expression in osteoblasts are unknown.

The MC3T3-E1/4 cell line is a pre-osteoblast cell line derived from murine (*Mus muscularis*) calvarial cells. Under appropriate growth conditions, MC3T3-E1/4 cells differentiate into mature osteoblasts and are capable of synthesising mineralised extracellular matrix [23]. MC3T3-E1/4 cells have also been shown to increase TRAP+ cell number, (indicative of osteoclast cell number) when co-cultured with bone marrow cells indicating they are capable of supporting osteoclastogenesis [24, 25]. They have also been shown to induce osteoclast activity *in vitro* [26].

We hypothesised that treatment of MC3T3-E1/4 cells with the anti-inflammatory LCPUFAs, GLA, EPA and DHA, would reduce membrane-bound RANKL expression whereas treatment with the pro-inflammatory LCPUFA would increase membrane-bound RANKL expression. To this end, the effect of AA, GLA, EPA and DHA on membrane-bound RANKL expression in cells under standard culture conditions was investigated. Secondly, the effect of GLA, EPA and DHA on membrane-bound RANKL expression in cells in which RANKL expression had been stimulated by PGE2 was assessed.

Methods

Materials

Gamma-linolenic acid, docosahexaenoic acid, arachidonic acid, eicosapentaenoic acid and prostaglandin E2 (P0409) were purchased from Sigma-Aldrich Co., New Zealand. Phenol red-free α-MEM (41061-029), foetal calf serum and RNase A (20mg/ml, 12091-021) were purchased from Invitrogen New Zealand Ltd. Biotinylated anti-mouse TRANCE (RANKL, CD254) antibody was purchased from BioLegend, San Diego, USA (catalogue # 510004). Phycoerythrein-conjugated streptavidin (streptavidin-PE)

was purchased from AbD Serotec, Oxford, UK. Phycoerythrein-conjugated CD61 (553347) and CD8a (553033) were purchased from BD Pharmingen, New Jersey, USA. The MC3T3-E1/4 cell linc was purchased from ATCC, Manassas, VA, USA (ATCC® number CRL-2593™).

Culture conditions

MC3T3-E1/4 pre-osteoblast cells were cultured in 6-well plates at a density of 3 x 10^5 cells/ml with two replicate wells per treatment within each experiment. Cells were cultured in phenol red-free α-MEM with 10% heat-inactivated foetal calf serum (FCS) and either carrier (ethanol, 0.05%) or LCPUFA (5μg/ml) with and without indomethacin (1μM) or PGE2 (10^{-8}M) for 48 hours at 37°C in a humidified atmosphere of 95% air/ 5% CO_2.

Measurement of OPG secretion

Following incubation, cell supernatant was collected, immediately frozen and stored at -80°C until analysis. Concentration of OPG in cell supernatants was determined using an OPG ELISA kit supplied by R&D Systems, Minneapolis, USA (catalogue # MOP00). Intra-assay coefficient of variation was 6.4%. Cell number was determined by crystal violet staining as previously described [27]. In short, after removal of media, cells were washed with PBS, fixed with 1% formaldehyde and incubated with 1% crystal violet for 60 minutes at 37°C. Following thorough washing, stain was extracted from cells with 0.2% Triton X-100 and absorbance read at 550nm using an ELx808 Ultra microplate reader (Bio-Tek Instruments Inc., Vermont, USA). Cell number in experimental wells was determined by normalising to the reading of a standard curve derived from a known number of cells per well. OPG concentration was expressed as amount of OPG per cell. Experiments were independently replicated at least three times.

Measurement of membrane-bound RANKL

Cells were washed twice in PBS then removed from the culture plate by gentle scraping with a rubber cell scraper. Cells were re-suspended in phenol red-free α-MEM/1% FCS and stained for cell surface antigen as described previously [28]. Approximately 150,000 cells were incubated with either biotinylated anti-mouse RANKL or isotype-matched irrelevant control (PE-CD8a) or positive control (PE-CD61) for 30 minutes at 4°C. Cells were washed with PBS. Streptavidin-PE was added to anti-mouse RANKL-

treated cells and cells were incubated for a further 30 minutes at 4°C. Cells were washed, fixed in 1% formaldehyde and analysed by flow cytometry using a FACSCalibur system and CellQuest software (BD Biosciences, San Jose, Ca, USA). Experiments were independently replicated at least three times.

Determination of Cell Cycle Stage

Cells were washed twice with PBS and trypsinised. Following resuspension in 50µl PBS, cells were treated with 600µl of ice-cold 70% ethanol and incubated on ice with continuous mixing in the dark for 1 hour. Cells were washed twice with PBS, resuspended in DNase-free RNase A (1mg/ml) and incubated in the dark for 15 minutes at room temperature. Propidium iodide (200µl, 100µg/ml) was added and cells incubated at 2°C for 2hrs. Cells were analysed by flow cytometry using a FACSCalibur system and CellQuest software (BD Biosciences, San Jose, Ca, USA) and cell cycle distribution determined by a previously described method [29]. Washed cells were also labelled with Annexin V-FITC (5µl/200,000 cells) to confirm detection of apoptotic/necrotic cells [29].

Statistical Analysis

All data conformed to the requirements of the general linear model. Results were analysed by one-way ANOVA with post-hoc Tukey-Kramer testing. A p-value of $\leq$ 0.05 was considered statistically significant.

Results

Membrane-bound RANKL expression in MC3T3-E1/4 cells

MC3T3-E1/4 cells are a heterogeneous mix in terms of cell size. As shown in **Figure 1**, two distinct sub-populations of MC3T3-E1/4 cells expressed RANKL. Visual examination of the cells at 100× microscopy revealed a single-cell suspension indicating that the two populations did not represent singlet and doublet cells. Basal RANKL expression was the same in the two cell populations with approximately 25-35% of cells expressing RANKL.

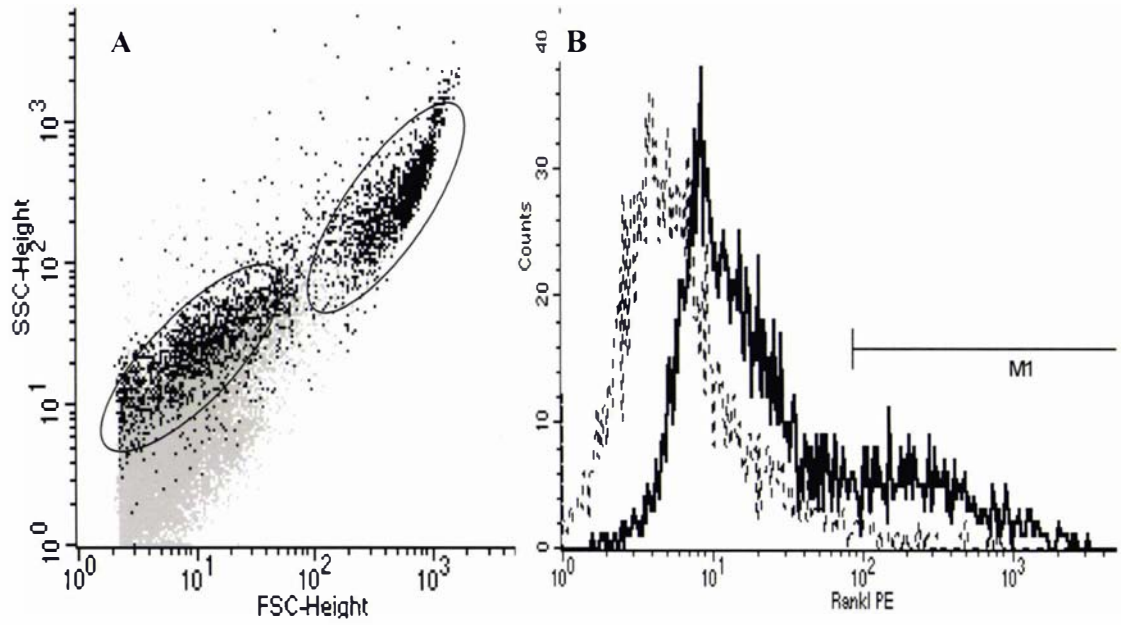

Figure 1 Detection of membrane bound RANKL by flow cytometry ***A*** Dot plot of MC3T3-E1/4 cells showing two distinct sub-populations of cells. Black cells are RANKL-positive cells, grey cells are RANKL-negative. FSC = forward scatter, SSC = side scatter. The greater the forward and side scatter, the larger the cell size. ***B*** Overlay of histogram showing PE (phycoerythrein) detection in MC3T3-E1/4 cells exposed to isotype-matched irrelevent control antibody (CD8a, dotted line) and RANKL (solid line).

Cell Cycle Stage of MC3T3-E1/4 cells

The population of cells with higher forward (FSC) and side scatter (SSC) characteristics ("larger cells") were determined by propidium iodide staining to be viable cells with approximately 80% in G0/G1 (diploid), 5-6% in S-phase and 11% undergoing mitosis (tetraploid). Less than 0.5% of large-sized cells were apoptotic or necrotic (defined as a sub-90 peak) (**Figure 2**). The smaller-sized cells were determined by propidium iodide to be apoptotic/necrotic (**Figure 2**). This was confirmed by localisation of Annexin V-FITC labelling in the smaller-sized cell population (not shown). The smaller-sized apoptotic/necrotic cell sub-population comprised approximately 10% of total (large + small) cells. Cell cycle stage and the percentage of apoptotic/necrotic cells relative to viable cells were unchanged with LCPUFA treatment (data not shown).

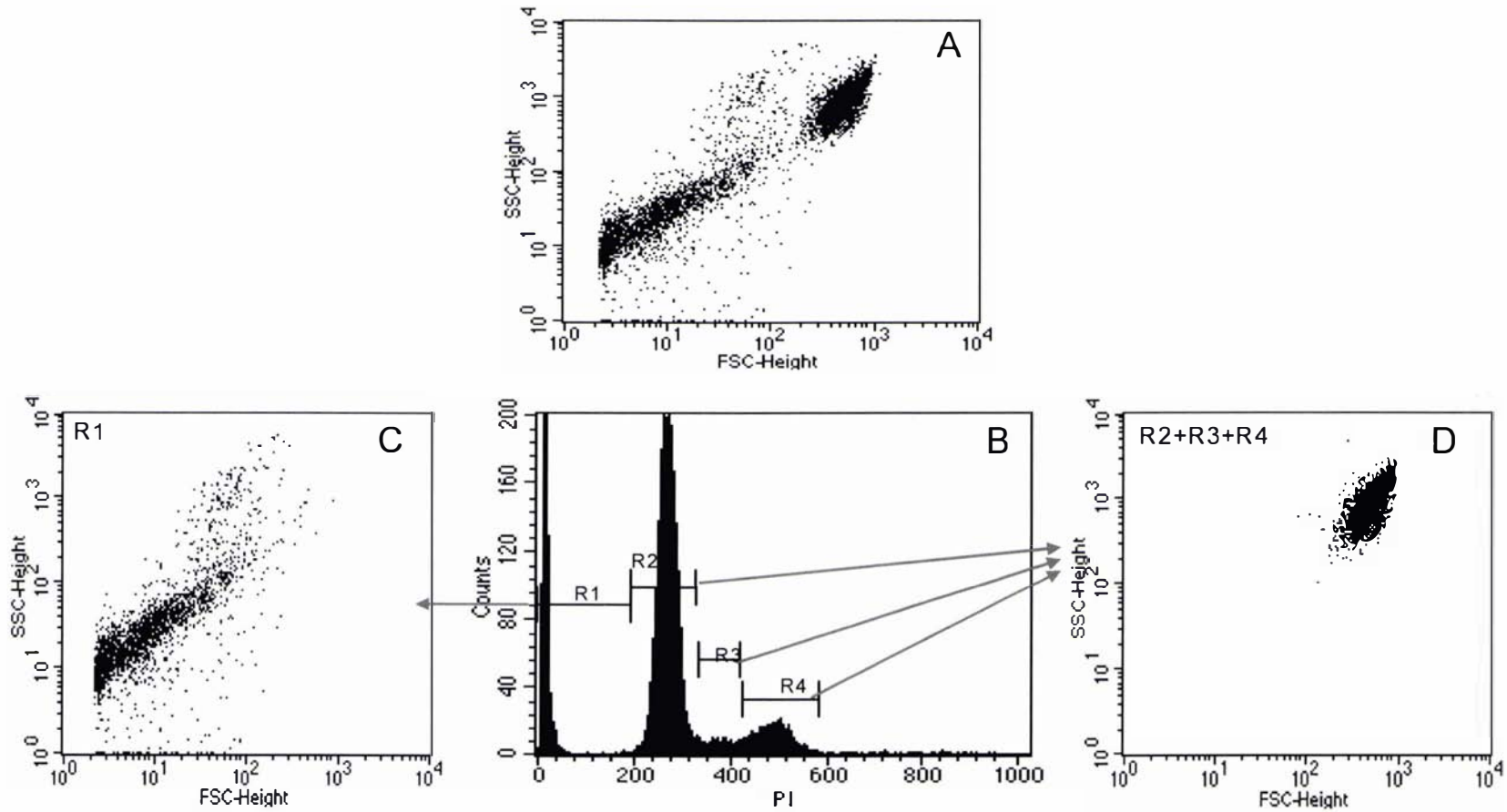

Figure 2 Cell cycle stage of MC3T3-E1/4 cells as determined by flow cytometry (A) Dot plot of MC3T3-E1/4 cells' scatter characteristics showing two distinct sub-populations of cells. (B) Cell cycle distribution of the total cell population (C) Back-gating to show cells in R1 (apoptotic/necrotic cells) and (D) Back-gating to show cells in R2+R3+R4 (G0/G1, S, G2/M stages of cell cycle respectively).FSC = forward scatter, SSC = side scatter. The greater the forward and side scatter, the larger the cell size.

Effect of LCPUFA treatment on membrane-bound RANKL expression in MC3T3-E1/4 cells

In the viable cells, EPA and DHA had no effect on basal RANKL expression. Both GLA and AA treatment were associated with a higher percentage of viable cells expressing RANKL (**Figure 3**). However, the percentage of RANKL-positive cells was not significantly different from controls following combined treatment with GLA or AA and the COX inhibitor indomethacin (**Figure 4**). The percentage of necrotic cells expressing RANKL was unchanged following 48hrs of treatment with AA or GLA.

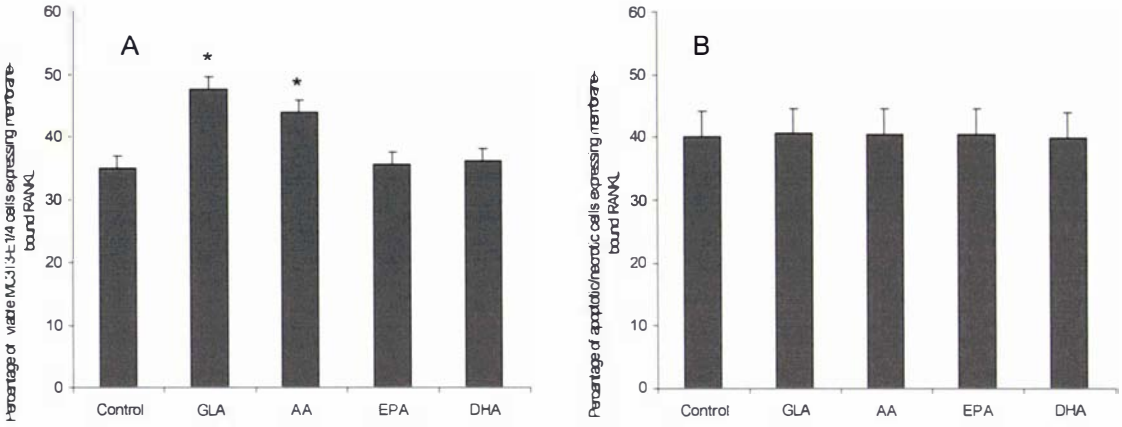

Figure 3 Percentage of (A) viable and (B) apoptotic/necrotic MC3T3-E1/4 cells expressing membrane-bound RANKL. Cells were treated for 48hrs with LCPUFAs (5μg/ml) and membrane-bound RANKL measured by flow cytometry. Groups marked with * were significantly different ($p<0.05$) from control. Results are the mean and SE of three independent experiments.

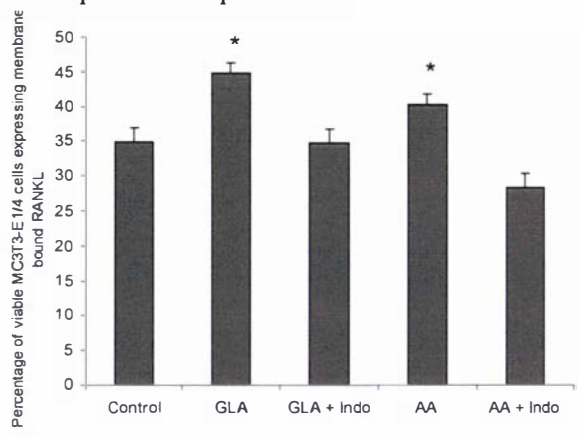

Figure 4 Percentage of viable MC3T3-E1/4 cells expressing membrane-bound RANKL following treatment with arachidonic acid (AA) or gamma-linolenic acid (GLA) and the cyclooxygenase inhibitor indomethacin (indo). Cells were treated for 48hrs with LCPUFAs (5μg/ml) with or without indomethacin (1μM). Membrane-bound RANKL expression was measured by flow cytometry. Groups marked with * were significantly different ($p<0.05$) from control. The percentage of viable cells expressing membrane-bound RANKL was significantly lower in GLA + Indo compared to GLA and in AA + Indo compared to AA. Results are the mean and SE of three independent experiments.

Next we examined the effect of EPA, DHA and GLA on RANKL expression and OPG secretion in cells in which RANKL expression had been stimulated by pro-inflammatory PGE2. As expected, PGE2 treatment increased the percentage of cells expressing RANKL (**Figure 5**). However, the percentage of RANKL positive cells was not significantly different from controls when cells were treated with PGE2 and either EPA or DHA. GLA had no discernible effect on RANKL expression in PGE2-treated cells (**Figure 5**).

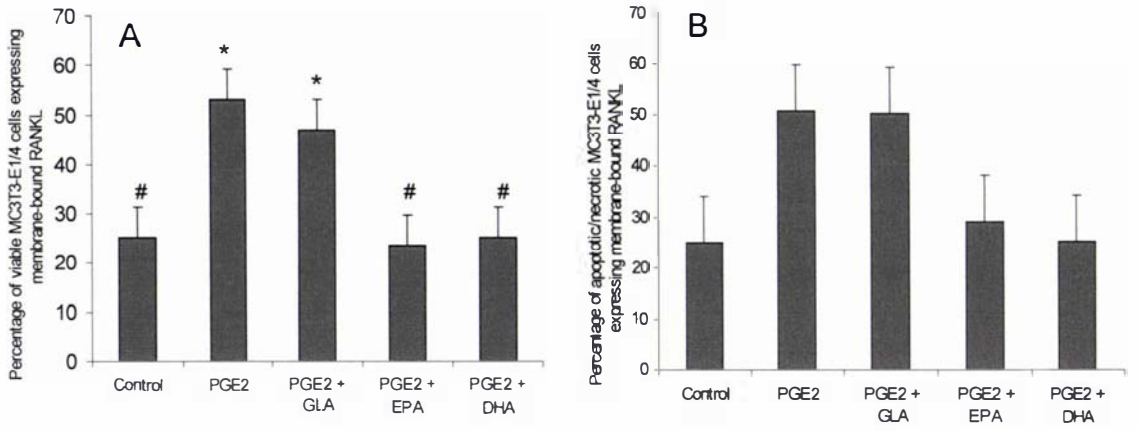

Figure 5 Percentage of (A) viable and (B) apoptotic/necrotic PGE2-exposed MC3T3-E1/4 cells expressing membrane-bound RANKL. Cells were treated for 48hrs with PGE2 (10^{-8}M) with or without LCPUFAs (5μg/ml). Membrane-bound RANKL expression was measured by flow cytometry. Groups marked with * were significantly different ($p<0.05$) from control. Groups marked with # were significantly different ($p<0.05$) from PGE2. Differences between groups in graph B failed to reach statistical significance. Results are the mean and SE of three independent experiments

Effect of LCPUFA treatment on OPG secretion by MC3T3-E1/4 cells

Treatment with AA or PGE2 reduced mean OPG concentration per thousand cells by approximately 20%. Treatment with DHA, EPA or GLA alone had no statistically significant effect on mean OPG secretion per thousand cells. Similarly, treatment with DHA, EPA or GLA in conjunction with PGE2 treatment had no statistically significant effect on mean OPG secretion per thousand cells (**Figure 6**).

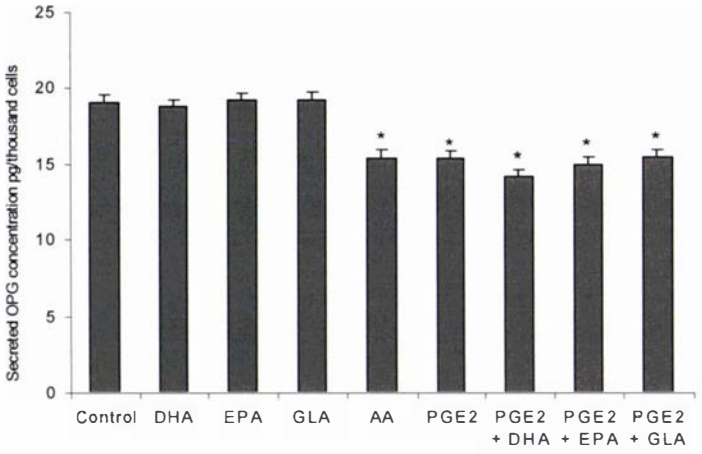

Figure 6 Osteoprotegerin (OPG) secretion (pg/thousand cells) by MC3T3-E1/4 cells Cells were treated for 48hrs with LCPUFAs (5μg/ml) with or without PGE2 (10^{-8}M). OPG concentration was measured in the cell supernatant by ELISA. Groups marked with * were significantly different ($p<0.05$) from control. Results are the mean and SE of three independent experiments.

Discussion

Membrane-bound RANKL was detectable on both viable and apoptotic/necrotic MC3T3-E1/4 osteoblast-like cells. Both the percentage of cells expressing membrane-bound RANKL and the amount of OPG secretion relative to the size of the cell population were significantly influenced by LCPUFA treatment.

Both AA and PGE2 treatments led to an increase in the proportion of cells expressing membrane-bound RANKL and a decrease in mean OPG secretion per cell. Treatment with AA may have been slower acting than treatment with PGE2 as membrane-bound RANKL expression was elevated in the apoptotic/necrotic cell sub-population 48 hours after exposure to PGE2 but not AA. This suggests that PGE2 treatment was able to influence membrane-bound RANKL expression in cells that were nearing the end of their life-cycle at the time of treatment whereas AA treatment may have had little effect on RANKL expression in these cells. Treatment with the COX inhibitor indomethacin resulted in loss of the stimulatory effect of AA on RANKL expression indicating that COX-derived products of AA rather than AA itself may be largely responsible for the increase in membrane-bound RANKL expression. Coetzee et al (2007) reported increased secretion of sRANKL and decreased secretion of OPG by MC3T3-E1/4 cells following treatment with either AA or PGE2 and the effects of AA were blocked by addition of indomethacin [16].

Overall findings from the present study and that of Coetzee et al (2007) suggest that COX-derived products of AA, but not AA itself, increase both membrane-bound and soluble RANKL expression in osteoblasts and decrease OPG secretion *in vitro*.

In the present study, GLA also increased the percentage of cells expressing membrane-bound RANKL. This effect was possibly a result of the activity of COX-derived products from GLA since the percentage of cells expressing membrane-bound RANKL was not different from non-LCPUFA treated controls when cells were co-incubated with GLA and indomethacin. GLA treatment did not further increase membrane-bound RANKL expression in PGE2-treated cells above that observed with PGE2 treatment alone. This may indicate that RANKL expression had been maximally stimulated in the PGE2-treated cell population or that PGE2 and GLA treatments interact such that one treatment can block the effect of the other on membrane-bound RANKL expression. In bone marrow culture, PGE1, which is a COX-derived product of di-hommogamma linolenic acid (DGLA), a reduction product of GLA, has been shown to have similar potency to PGE2 in promoting osteoclastogenesis [30]. Results from the present study suggest that the RANKL pathway may contribute to the induction of osteoclastogenesis by PGE1.

Treatment of MC3T3-E1/4 cells cultured under standard conditions with either DHA or EPA had no effect on the percentage of RANKL-positive cells or mean OPG secretion/cell. However, in MC3T3-E1/4 cells stimulated with PGE2, both DHA and EPA prevented the PGE2-induced increase in the percentage of RANKL positive cells. Previously, increased dietary intake of fish oil (rich in EPA and DHA) has been shown to prevent the ovariectomy-induced increase in membrane-bound RANKL expression in murine T-cells [20]. Treatment with either DHA or EPA has been shown to inhibit osteoclastogenesis in bone marrow culture [20]. Several intervention studies have demonstrated that dietary consumption of n-3 LCPUFAs, particularly the very-long chain n-3 PUFAs such as EPA and/or DHA, provides some protection against bone loss post-ovariectomy in rodents [20, 21, 31-34] and post-menopause in women [22]. Previously, the mechanism by which n-3 LCPUFAs protected against bone loss was largely attributed to competitive inhibition of n-6 LCPUFA metabolism, in particular the prevention of COX-mediated synthesis of PGE2 [35]. Results of the present study suggest that at least *in vitro*, EPA and DHA have

specific effects on osteoblasts independent from those involving inhibition of AA metabolism. *In vitro*, EPA and DHA may inhibit PGE2 activity at least to some extent. The observation that EPA and DHA prevented the PGE2-induced increase in membrane-bound RANKL expression in osteoblasts in the present study, as well as the previous finding that fish oil supplementation prevented the ovariectomy-induced increase in T-cell RANKL expression in rats [20] suggests EPA and DHA may reduce RANKL-initiated osteoclastogenesis. This may have implications for the treatment of inflammation-induced bone loss. The effects of EPA and DHA on osteoclastogenesis and bone resorption *in vivo* remain to be determined.

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CHAPTER 4

Digestibility of daidzein and genistein and urinary excretion of the isoflavones and their metabolites in the ovariectomised rat

Both long chain polyunsaturated fatty acids and phytoestrogens may minimise ovariectomy-induced bone loss. It is of interest to determine if combined dietary supplementation with DHA and phytoestrogens has a greater bone-protective effect than either treatment alone. The bioavailability of phytoestrogens varies considerably depending on a variety of factors including the chemical form of the phytoestrogen, the presence of other dietary factors and the phenotype of the individual consuming the supplement. As a preliminary step before examining the effects of combined phytoestrogen and DHA treatment on bone mass, the digestibility of the phytoestrogen supplements to be used and the effect of DHA on phytoestrogen digestibility and metabolism in the ovariectomised rat were determined.

Abstract

Consumption of the soyabean isoflavones, genistein and daidzein, may minimise the risk of developing postmenopausal osteoporosis. Similarly, consumption of the n-3 fatty acid docosahexaenoic acid (DHA) may have therapeutic value in combating postmenopausal osteoporosis. The aim was to determine the bioavailability of dietary genistein and daidzein in ovariectomised rats, a model for postmenopausal osteoporosis. The effect of co-supplementation with DHA on isoflavone bioavailability and metabolite formation was also assessed. Twenty female rats were ovariectomised and randomised into four groups. Animals were fed a diet containing either genistein or daidzein (0.026% of diet) with or without DHA (0.6% of diet) for four weeks. Urine and faeces were collected quantitatively for 6-days in the final week of the study. Animals were euthanased and plasma and ileal digesta collected. Genistein, its metabolite 4-ethylphenol; daidzein and its metabolite equol were present in plasma at concentrations of 24 nmol/L, 67 nmol/L, 17 nmol/L and 18 nmol/L respectively, indicating that the isoflavones were metabolised and that both the isoflavones and their metabolites were absorbed. The main mode of excretion of the isoflavones was via urine. The major excretory form of genistein was 4-ethylphenol and of daidzein was equol. Ileal digestibility of genistein was 93% compared to 31.6% for daidzein. Faecal digestibility of genistein was also greater than that of daidzein (99.9% compared to 77.5%). Inclusion of DHA in the diet did not influence genistein or daidzein bioavailability or metabolite formation.

Introduction

Epidemiological studies suggest that soyabean isoflavone consumption may aid in minimising post-menopausal bone loss in women, consequently reducing the incidence of osteoporosis [1-3]. However, intervention trials in both post-menopausal women and ovariectomised animals have yielded mixed results [4-19]. Interpretation of these results is confounded by the varying forms of isoflavone supplement used (aglycone or glycoside) as well as differences in other dietary constituents such as protein, fat or fibre, which may influence isoflavone uptake and/or metabolism [20]. Isoflavone metabolism and absorption can vary substantially depending on the form in which it is consumed [21]. There is a need to determine the bioavailability of

isoflavones and in some cases, of their more potent metabolites, in any supplement or food used in intervention trials, to determine the efficacy of isoflavone supplementation as a means of optimising health.

The metabolism of isoflavones is complex and involves endogenous enzymes, the gut microflora, as well as enterohepatic cycling. Recently, metabolic pathways for the two soya isoflavones genistein and daidzein have been proposed [22-24] and the resultant metabolites qualitatively identified in biological samples [23, 25]. Generally, studies reporting the bioavailability of these two isoflavones have focused solely on measuring uptake of the parent compounds and equol, a bioactive metabolite of daidzein. However at least one of the genistein metabolites, 4-ethylphenol, is also bioactive [26]. Little is known about the uptake of this, and other metabolites of either genistein or daidzein. In the presently reported study we aimed to quantify the known and presumed genistein metabolites namely dihydrogenistein, 4-ethylphenol, 4-hydroxyphenyl-2-propionic acid, 1,3,5-trihydroxybenzene and 1,3,5-trihydroxybenzoic acid as well as the daidzein metabolites dihydrodaizdein, equol and o-desmethylangolensin (o-dma) in plasma, ileal digesta, urine and faeces in genistein- and daidzein-supplemented ovariectomised rats. The ovariectomised rat is a FDA-approved animal model for postmenopausal osteoporosis and uptake of the full range of isoflavone metabolites has not previously be quantified in this commonly used model. The study also allowed us to determine quantitative input/output balances for genistein, daidzein and their metabolites.

A second objective of the study was to determine if dietary inclusion of the n-3 fatty acid, docosahexaenoic acid (DHA), also a potential anti-osteoporotic agent [27, 28], would alter the uptake or metabolism of either isoflavone. Slavin *et al* (1998) observed that equol production was significantly enhanced by increased dietary fatty acids [29]. Whether changing the degree of un-saturation of dietary fat influences isoflavone metabolism is unknown. As both isoflavones and DHA are potential therapeutics for osteoporosis prevention or treatment, and combination n-3 fatty acid and isoflavone therapies have been proposed [30], there is interest in determining if there are antagonistic, additive or synergistic interactions between the two treatments and whether DHA influences isoflavone metabolism or bioavailability.

Methods

Materials

Docosahexaenoic acid ethyl ester (80% purity) was purchased from Sanmark LLC, USA (product number 01177B-E80). Genistein (>95%) and daidzein (>96%) were purchased from LC Laboratories, USA. β-glucuronidase/arylsulfatase (G-7017) and all standards used for GC/MS analysis (except for 4-hydroxyphenyl-2-propionic acid) were purchased from Sigma Aldrich, Munich, Germany. The standard for 4-hydroxyphenyl-2-propionic acid (98%) was purchased from Acros Organics, Geel, Belgium (catalogue number 302680050). All other chemicals were of analytical grade and were purchased from Sigma-Aldrich, Munich, Germany.

Animals

The study was approved by The Massey University Animal Ethics Committee (Approval number 05/97) and was conducted in accordance with the principles of laboratory animal care [31]. Twenty 7-month old female Sprague-Dawley rats were obtained from the Small Animal Production Unit, Massey University. All animals underwent bilateral ovariectomy performed under general anaesthetic (isofluorane).

Animals were initially individually housed in shoebox cages. For the final 14 days of the trial, animals were transferred to individual, stainless steel wire-bottomed metabolic cages to allow for the separation and collection of urine and faeces. Throughout the trial, animals were housed in a dedicated room maintained at 22°C (± 2°C) and with a 12h/12h light/dark cycle.

Diets

Animals were gradually introduced to a nutritionally-balanced semi-synthetic maintenance diet over a period of two weeks prior to undergoing ovariectomy (week -2 to week 0). The diet formulation was based on AIN93M [32] with vitamins and minerals added as necessary to compensate for the nutrient content of local ingredients [33]. The type of oil in the diet was also altered from soybean oil (as stipulated for AIN93M) to corn oil as soybean oil is a source of n-3 fatty acids. Titanium dioxide was added to the diet (5g/kg diet) as an indigestible marker to allow quantification of digesta and faecal phytoestrogen and metabolite contents.

Following ovariectomy, animals were assigned at random to one of four experimental groups (n=5 per group). The maintenance diet was supplemented with 0.026% genistein (2 groups) or daidzein (2 groups). To one genistein-supplemented diet (GENFAT) and one daidzein-supplemented diet (DAIFAT), docosahexaenoic acid ethyl ester (DHA) was added at a dose of 0.6% of diet and the amount of corn oil in the diet was reduced to 3.4%. The remaining genistein (GEN) and daidzein (DAI) supplemented diets contained corn oil (4% of diet) as the sole source of fat. Total fat and energy content of all diets was the same. Samples of the diets were frozen (-20°C) and finely ground before chemical analysis. The ingredient compositions are given in **Table 1**.

Table 1 *Ingredient composition (% air-dry weight) of the experimental diets*

	Percentage of Diet			
	GEN	***GENFAT***	***DAI***	***DAIFAT***
Cornstarch	59.174	59.174	59.174	59.174
Sodium Caseinate	14	14	14	14
Sucrose	6	6	6	6
Cellulose [a]	5	5	5	5
Vitamins [b]	5	5	5	5
Minerals [c] (excluding calcium)	5	5	5	5
Calcium Carbonate	1.3	1.3	1.3	1.3
Corn Oil [d]	4	3.4	4	3.4
Docosahexaenoic acid ethyl ester [d]	0	0.6	0	0.6
Titanium Dioxide	0.5	0.5	0.5	0.5
Genistein	0.026	0.026	0	0
Daidzein	0	0	0.026	0.026

[a] Vitacel L600, Swift NZ Ltd

[b] Supplying (mg/kg diet) retinol acetate 5.0, $_{DL}$-α-tocopherol acetate 200.0, menadione 3.0, thiamine hydrochloride 5.0, riboflavin 7.0, pyridoxine hydrochloride 8.0, D-pantothenic acid 20.0, folic acid 2.0, nicotinic acid 20.0, D-biotin 1.0, myo-inositol 200.0, choline chloride 1500; (µg/kg diet) ergocalciferol 25.0, cyanocobalamin 50.0.

[c] Supplying (g/kg diet) chloride 7.79, magnesium 1.06, phosphate 4.86, potassium 5.24, sodium 1.97; (mg/kg diet) chromium 1.97, copper 10.7, iron 424, manganese 78.0, zinc 48.2; (µg/kg diet) cobalt 29.0, iodine 105.0, molybdenum 152.0, Selenium 151.0

[d] Total fat content of all diets was 4%.

Feeding Regimen

De-ionised water was freely available to the animals at all times. For the initial 10 days following ovariectomy, animals had unrestricted access to their respective diets. After the post-surgery recovery period (10 days), animals were trained to consume one meal per day. During this period, animals had unrestricted access to their respective diets for 3 hours per day (0700-1000), after which time food was removed.

Two weeks following ovariectomy, animals were placed in metabolic cages for a 1-week acclimatisation period followed by a 6-day period of faeces and urine collection. The design of the metabolic cages was such that coprophagy was prevented.

Metabolic Balance and Sample Collection

Rat body weight was recorded immediately prior to and following the sample collection period. All faeces and urine were collected separately on a daily basis and daily food intake was recorded. Hydrochloric acid (1M) was added to the faeces and urine collection tubes (1ml per tube) prior to sample collection to prevent continued microbial degradation of the isoflavones and metabolites in these samples post-excretion. Excreta were frozen (-20°C) and faecal samples were subsequently freeze-dried and stored frozen (-20°C).

Immediately following the meal on day 7 of the metabolic balance period, animals were deeply anaesthetised via intra-peritoneal injection (25G x 5/8" needle and 1ml syringe) of 0.1ml/kg body weight of acepromazine, ketamine, xylazine and sterile H_2O (2 : 5 : 1 : 2) and were subsequently exsanguinated by cardiac puncture (19G x 1 ½" needle and 5ml syringe). Blood was collected in EDTA-containing vacutainers and immediately centrifuged at 2000rpm for 10 minutes. Plasma was stored at -80°C for later analysis. Digesta from the terminal 20cm of the ileum immediately distal to the ileo-caecal valve were collected by flushing with 10ml of milli Q water using a 10ml syringe attached to a mouse gavage needle. Digesta were frozen at -20˚C and subsequently freeze-dried prior to chemical analysis.

Sample Extraction

Urine

For each animal, total urine collected over the 6-day period was pooled and filtered through Whatman #4 filter paper to remove any contaminating particulate matter. Samples were mixed to ensure homogeneity and isoflavones were extracted as described earlier with slight modifications [34]. Urinary creatinine concentration was determined by the Jaffe method [35]. Urine samples were buffered to pH5 with 1ml of 0.125M sodium acetate. Tyrosol (25µmol/mg creatinine) was added as an internal

standard followed by 125µl β-glucuronidase/arylsylfate and the samples were incubated overnight at 37°C. Samples were then acidified with 5 N HCl to a pH < 2. Samples were extracted twice, firstly with 6ml ethyl acetate and then with 3ml diethyl ether. An excess of anhydrous Na_2SO_4 was added (approximately 2 mg) to remove any water that may have still been present in the sample. This volume was vortexed for 30 seconds and centrifuged for 5 minutes. The solution was decanted from the pellet and dried under a nitrogen stream. The dry extract was derivatised at 90 °C for 30 minutes with 22.6 µL bis(trimethylsilyl) trifloroacetamide (BSTFA)/µmol creatinine, 4.5µL pyridine/µmol creatinine and 4.5 µL trimethylchlorosilane (TMCS)/µmol creatinine.

Ileal Digesta and Faeces

Freeze-dried ileal digesta and faecal samples were finely ground using a Breville coffee bean grinder. Digesta (50mg) were mixed with 1mL 0.125M sodium acetate buffer (pH 5). For faecal analysis, 250mg faeces and 2mL 0.125M sodium acetate buffer (pH 5) were used. In both cases 100ul of a 0.5mg/ml tyrosol solution was added as an internal standard. Samples were deconjugated, acidified, extracted and derivatised as for the method for urine samples.

Plasma

To 200µl of plasma, 100µl of 0.5mg/ml tyrosol solution was added. As isoflavones are present predominately as conjugates rather than aglycones in blood [36] and are tightly bound to various blood proteins [37], plasma samples were treated with 200µl 100% acetonitrile to denature binding proteins, centrifuged at 3000rpm for 5 minutes and the clear supernatant collected prior to enzymatic deconjugation. Deconjugation, acidification, extraction of isoflavone metabolites and derivatisation was conducted as described for urine.

GC/MS analysis of isoflavone metabolites

An Agilent 6890N gas chromatograph system with an HP-5 capillary column (0.25mm x 30m x 0.25µm) coupled to a 5973 mass spectrometer was used for the analysis. Injection volume was 1.0µl with a split ratio of 10:1 and a helium flow rate of 1ml/min. Injection inlet temperature was 250°C. Initial oven temperature was

120°C maintained for 2 minutes. Temperature was ramped at 10°C/minute to 320°C where it was maintained for a further 2 minutes. Total run time was 24 minutes.

Quantification of Titanium Dioxide in Faeces and Ileal Digesta

Faecal concentration of the indigestible marker titanium dioxide was determined by colourimetry following sulphuric acid digestion as previously described [38]. Due to the relatively small quantity of ileal digesta available for analysis, the concentration of titanium was determined in the digesta residue remaining after isoflavone extraction by ICP-MS following sulphuric acid digestion. Concentration of titanium dioxide in digesta was calculated based on the respective molecular weights of titanium and oxygen and corrected for the quantity of isoflavones extracted.

Data Analysis

Ileal and faecal genistein, daidzein and metabolite concentrations with respect to genistein and daidzein intake were determined as follows:

Concentration of compound (mg/g DMI) =
[Compound in digesta or faeces (mg/g DM) × TiO_2 in diet (mg/g DM)] / TiO_2 in digesta or faeces (mg/g DM)

Where:
Compound = genistein, daidzein or metabolite
DMI = Dry matter Intake
DM = Dry matter

Ileal and faecal daidzein and genistein digestibility were determined by:

Ileal or faecal digestibility of compound (%) =
([Dietary genistein or daidzein (mol/g DMI)] – [ileal or faecal genistein or daidzein and their metabolites (mol/g DMI)]) / [Dietary genistein or daidzein (mol/g DMI)] × 100%

Statistical Analysis

Data were analysed using Minitab version 14 (Minitab Inc., Pennsylvania, USA). Results are reported as mean ± standard error of mean (SEM). Analysis of variance using the general linear model with post-hoc Tukey testing was used for comparisons between groups. A p-value of ≤ 0.05 was considered statistically significant.

Results

There were no statistically significant differences among the treatment groups in terms of mean daily food intakes. Average food intakes for the 6-day balance period (g/rat/day ± SE) were DAI 11.0 (± 0.7), DAIFAT 10.1 (± 0.7), GEN 11.0 (± 0.7), GENFAT 10.8 (± 0.7). Rats gained body weight over the 6-day balance period. There were no statistically significant differences among groups in weight gain or final body weight. Body weight gains (g/rat/6-days ± SE) were DAI 18.0 (± 3.3), DAIFAT 14.9 (± 3.3), GEN 18.2 (± 3.3) and GENFAT 20.0 (± 3.3). Final rat body weights (g ± SE) were DAI 303.0 (± 8.5), DAIFAT 297.4 (± 8.5), GEN 291.1 (± 8.5) and GENFAT 311.5 (± 8.5).

The presence of DHA in the diet had no statistically significant effect on either the ileal or faecal digestibility or the plasma or urine concentrations of the unmetabolised isoflavones or isoflavone metabolites (data not shown).

Isoflavones in ileal digesta, plasma, urine and faeces

The daidzein metabolites analyzed for were equol, o-dma and dihydrodaidzein. The concentrations of daidzein and its metabolites in ileal digesta, plasma, urine and faeces are given in **Table 2**. Equol was the main metabolite detected in all biological samples measured from daidzein-fed animals although small amounts of o-dma and 2-dehydro – o-dma were present in ileal digesta and urine respectively. Dihydrodaidzein, an intermediate product in daidzein metabolism, was also detected in ileal digesta and urine.

The metabolites of genistein which have been previously proposed [24] and which were analysed for in this study were: 4-ethylphenol, 4-hydroxyphenyl-2-propionic acid, dihydrogenistein, 1,3,5-trihydroxybenzene and 1,3,5-trihydroxybenzoic acid.

The latter two metabolites were not detected in any sample. The main genistein metabolite present in ileal digesta, plasma and urine was 4-ethylphenol. The latter metabolite was not detected in faeces. The only genistein metabolite detected in faeces was 4-hydroxyphenyl-2-propionic acid. This was also present in low concentrations in ileal digesta and urine.

Table 2 *Concentrations of genistein and known genistein metabolites, and daidzein and known daidzein metabolites in ileal digesta, plasma, urine and faeces*

DIET		***DIGESTA (μmol/kg DMI)***	***PLASMA (nmol/L)***	***URINE (μmol/L)***	***FAECES (μmol/kg DMI)***
Genistein	Genistein	65.2 (11)	24 (15)	89 (29)	Trace
	Dihydrogenistein	ND	ND	4.81×10^{-3} (4.44×10^{-3})	ND
	4-ethylphenol	1.83 (0.56)	67 (19)	340 (93)	ND
	4-hydroxyphenyl-2-propionic acid	0.265 (0.25)	ND	3.9 (1.6)	1.05 (0.69)
Daidzein	Daidzein	260 (25)	17 (13)	93 (21)	7.3 (3.6)
	Dihydrodaidzein (keto + enol)	trace	ND	5.94×10^{-2} (2.24×10^{-2})	ND
	o-dma	4.54×10^{-4} 4.54×10^{-4}	ND	ND	ND
	2-dehydro – o-dma	ND	ND	6.27×10^{-2} (1.86×10^{-2})	ND
	Equol	440 (59)	18 (4.9)	280 (56)	230 (66)

Ovariectomised rats consumed a diet supplemented with either genistein or daidzein (0.26mg/g DMI) for 4 weeks. Following euthanasea, genistein, daidzein and their known metabolites were measured in ileal digesta, plasma, urine and faeces by GC/MS. Results are expressed as mean with SE in ( ).
ND = Not detectable
DMI = Dry matter intake
o-dma = o-desmethylangolensin

Daidzein and Genistein Digestibility

The apparent ileal digestibility of daidzein was significantly lower than that of genistein ($p<0.0001$) (**Table 3**). Likewise, the faecal digestibility of daidzein was also significantly lower than that of genistein ($p<0.0001$).

Table 3 *Ileal and faecal digestibility of daidzein and genistein*

	Daidzein	***Genistein***	*Significance*
Ileal digestibility (%)	32.0 (7.9)	93.0 (1.1)	***
Faecal digestibility (%)	77.5 (6.5)	99.9 (0.06)	**

Ovariectomised rats consumed a diet supplemented with either genistein or daidzein (0.26mg/g DMI) for 4 weeks. Following euthanasea, genistein, daidzein and their known metabolites were measured in ileal digesta, and faeces by GC/MS.

** $p \leq 0.01$

*** $p \leq 0.0001$

DMI = Dry matter intake

The percentage disappearances of dietary genistein and daidzein from the digestive tract are shown in **Figure 1**.

Input/Output Balances

By averaging genistein and daidzein intakes and excretions over a 24hr period it is possible to estimate the extent to which determined excretion accounted for dietary isoflavone intake. To this end, average 24-hour input/output balances were calculated for genistein and daidzein (**Table 4a**). It should be noted that this study was not designed to assess the pharmacokinetics of genistein or daidzein uptake and excretion, therefore the input/output balances do not provide information with regard to the timing of excretion of the isoflavones relative to isoflavone consumption during the 6-day balance period.

"Input" was calculated as the average daily dietary intake of genistein or daidzein (moles) during the 6-day balance. Total faecal and urinary excretions of genistein, daidzein and their respective metabolites during the 6-day balance were calculated based on the molar concentrations of the compounds in urine and faeces and the total volume/mass of urine or faeces excreted over the period. Mean faecal and urinary isoflavone and metabolite excretions (moles) per 24hr period, "urinary and faecal outputs," were subsequently calculated. For all metabolites, metabolite formation from the parent isoflavone is on a mole per mole basis (i.e. 1 mole of parent isoflavone yields one mole of metabolite).

An estimate of the total blood volume of each animal was derived from published values [39] based on average rat body weight. Assuming that plasma constitutes 40-

50% of whole blood, an estimated total plasma volume of 9.75ml per animal was used. Average 24-hour plasma isoflavone and metabolite content was determined by multiplying the concentration of each compound detected in plasma by the estimated total plasma volume.

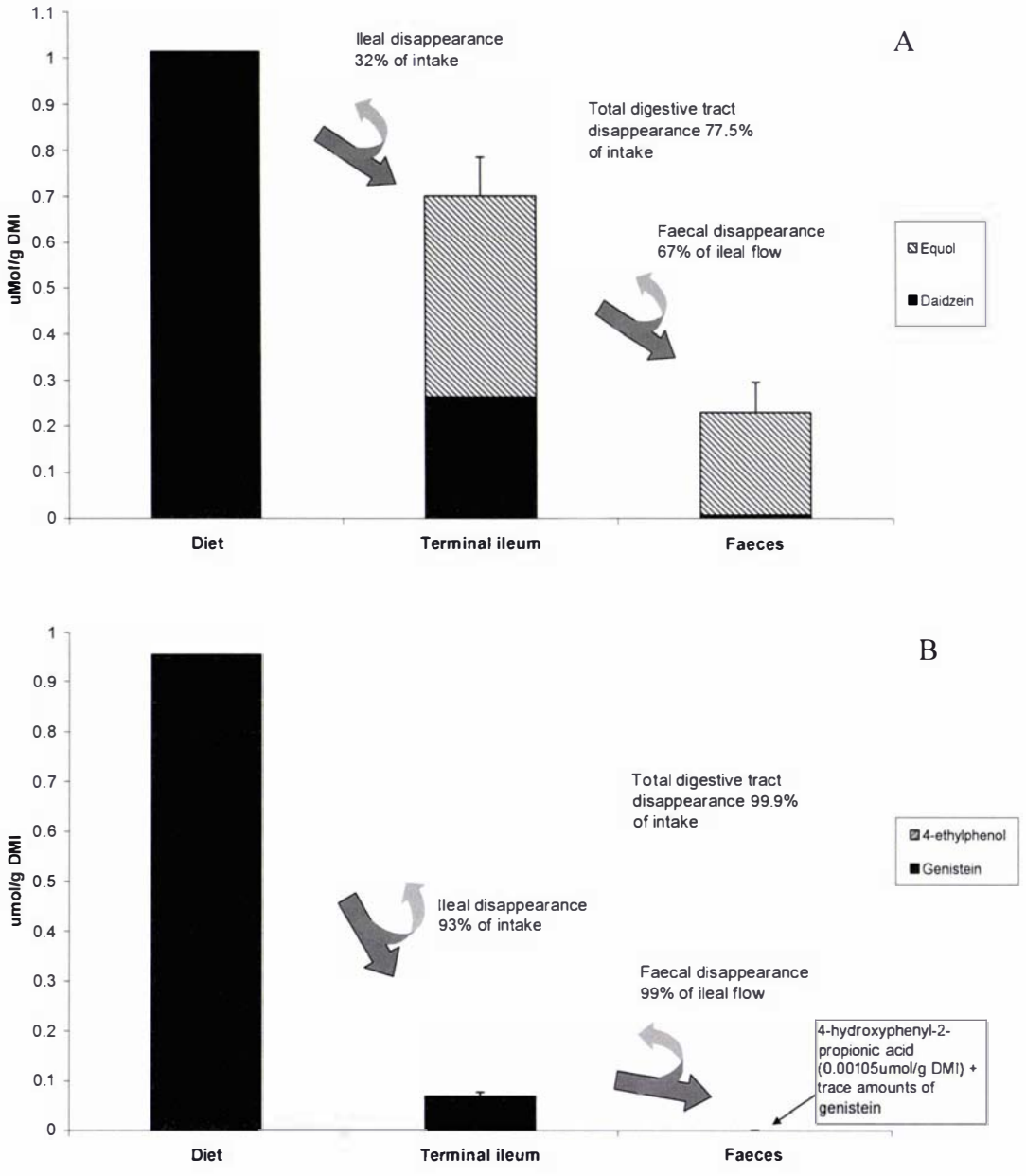

Figure 1 Diet, ileal digesta and faecal contents of unmetabolized daidzein (***A***) and genistein (***B***) and their known metabolites following dietary supplementation of ovariectomised rats. o-dma and trace amounts of dihydrodaidzein were detected in digesta from daidzein supplemented animals. The metabolite 2-dehydro-o-dma was also present in very low concentrations in urine from daidzein-supplemented animals (refer table 2). The genistein metabolite 4-hydroxyphenyl-2-propionic acid was detected in low concentrations in digesta from genistein-supplemented animals (refer table 2).

On a mol/mol basis, 24hr urinary plus faecal excretion of genistein and its known metabolites represented 43.9% of average 24-hour dietary genistein intake. Similarly, 24hr excretion of daidzein and its known metabolites represented 53.8% of average 24-hour dietary daidzein intake. Excretion of both genistein and daidzein was largely by way of urine **(Table 4a)**.

Table 4a *Input/Output balance (24hr) for genistein and daidzein in ovariectomised rats.*

INPUT (Diet)		**OUTPUT (Excretion)**				
	Quantity (µmol/ 24hr)		**Excretion in urine (µmol/ 24hr)**	**Excretion in urine as a % of genistein intake (Mol/Mol%)**	**Excretion in faeces (µmol/ 24hr)**	**Excretion in faeces as a % of genistein intake (Mol/Mol%)**
Genistein	10.131 (0.390)	Genistein	1.086 (0.335)	10.14 (3.01)	Trace	
		4-ethyl-phenol	4.18 (1.16)	40.2 (11.1)	ND	
		4-hydroxy phenyl-2-propionic acid	0.0487 (0.0188)	0.455 (0.172)	0.000646 (0.000434)	0.00683 (0.00446)
		Dihydro-genistein	0.0545 (0.0498)	0.463 (0.417)	ND	
		TOTAL	5.37	51.2	0.000646	0.00683
TOTAL INPUT 10.131 µMol		**TOTAL OUTPUT (URINE + FAECES) 5.37 µMol, 51.2% of mean 24hr genistein intake**				
INPUT (Diet)		**OUTPUT (Excretion)**				
Daidzein	10.833 (0.385)	Daidzein	0.928 (0.186)	8.86 (2.03)	0.057 (0.0282)	0.53 (0.264)
		Equol	3.015 (0.820)	27.47 (7.36)	1.564 (0.430)	14.59 (4.02)
		Dihydro-daidzein	0.555 (0.209)	5.41 (2.08)	ND	
		o-dma	ND		ND	
		2-dehydro-o-dma	0.647 (0.201)	5.85 (1.82)	ND	
		TOTAL	5.15	47.59	1.62	15.12
TOTAL INPUT 10.833µMol		**TOTAL OUTPUT (URINE + FAECES) 6.77 µMol, 62.7% of mean 24hr daidzein intake**				

Ovariectomised rats consumed a diet supplemented with either genistein or daidzein (2.74mg/24 hrs) for 4 weeks. Following euthanasea, genistein, daidzein and their known metabolites were measured in urine and faeces by GC/MS and mean 24 hr intake (input) and excretion (output) were calculated. Results are expressed as mean with SE in ( ).

Note: The difference between average 24-hour input and average 24-hour output (excretion) is 4.761µmol (49% of intake) for genistein and 4.063µMol (37% of intake) for daidzein and represents the amount of intake unaccounted for.

ND = Not detectable

At a given point in time, circulating levels of genistein and its known metabolites in plasma represented 0.08% of average 24hr genistein intake whereas circulating levels

of daidzein and its known metabolites represented 0.03% of average 24-hour daidzein intake (**Table 4b**).

Table 4b *Estimated plasma quantities of genistein and daidzein and their metabolites*

Diet		Quantity (nmol)^	Quantity in plasma as a % of genistein intake (Mol/Mol%)
Genistein	Genistein	0.227 (0.142)	0.02 (0.001)
	4-ethylphenol	0.634 (0.183)	0.06 (0.002)
	4-hydroxyphenyl-2-propionic acid	ND	
	Dihydrogenistein	ND	
	TOTAL IN PLASMA 0.860 nMol, 0.08% of mean 24hr genistein intake		
Daidzein	Daidzein	0.142 (0.109)	0.02 (0.001)
	Equol	0.152 (0.044)	0.02 (0.005)
	Dihydrodaidzein	ND	
	o-dma	ND	
	2-dehydro-o-dma	ND	
	TOTAL IN PLASMA 0.103 nMol, 0.03% of mean 24hr daidzein intake		

Ovariectomised rats consumed a diet supplemented with either genistein or daidzein (2.74mg/24 hrs) for 4 weeks. Following euthanasea, genistein, daidzein and their known metabolites were measured in plasma by GC/MS. Results are expressed as mean with SE in ( ).
^ Based on an estimated total plasma volume of 9.75ml.

ND = Not detectable
o-dma = o-desmethylangolensin

Discussion

The study had two primary objectives. Firstly to determine the amounts of daidzein, genistein and their metabolites in digesta, faeces, plasma and urine to demonstrate the degree of bioavailability in the ovariectomised rat model and secondly to determine if the inclusion of DHA in the diet affects the uptake or metabolism of daidzein or genistein in the ovariectomised rat model.

Endogenous mammalian enzymes are believed to be incapable of catabolising daidzein and consequently the formation of equol and o-dma (via the intermediate dihydrodaidzein) occurs due to metabolism by the gut microflora [40]. Although an

estimated 50-70% of humans are incapable of producing equol [41], presumably due to a lack of daidzein-metabolising gut bacteria [42], all rats are believed to be equol-producers [43]. In the present study, equol production was observed in all daidzein-fed rats and equol was present in relatively high amounts in ileal digesta, plasma, urine and faeces. Small amounts of o-dma or 2-dehydro – o-dma were also detected in ileal digesta and urine.

In contrast to daidzein, endogenous mammalian enzymes appear to be capable of metabolising genistein. Coldham *et al*, (2002) proposed two pathways for the biotransformation of genistein. The end-products of one pathway being 4-ethylphenol and 1,3,5-trihydroxybenzoic acid and of the other being 4-hydroxyphenyl-2-propionic acid and 1,3,5-trihydroxybenzene . In the present study, 4-ethylphenol was the main metabolite of genistein detected in plasma, urine and ileal digesta although small amounts of 4-hydroxyphenyl-2-propionic acid were also detected in both digesta and urine. Studies in sheep have also identified 4-ethylphenol as the main genistein metabolite in urine [44]. The major genistein metabolite excreted in faeces in the present study was 4-hydroxyphenyl-2-propionic acid which has previously been identified as the end-product of faecal and caecal microflora-mediated genistein metabolism [24]. We did not detect 4-ethylphenol in faeces and similarly Coldham *et al*, (2002) found no evidence of 4-ethylphenol production by either human or rat caecal microflora [24]. This suggests that endogenous enzymes in the stomach and/or small intestine favour conversion of genistein to 4-ethylphenol whereas genistein metabolism by the gut microflora largely results in 4-hydroxyphenyl-2-propionic acid production. Although 4-ethylphenol has no oestrogenic activity, it is bioactive and has been shown to stimulate prostaglandin $F_{2\alpha}$ synthase-like 2 (PGFSL2) gene expression in bovine endometrium [26]. As prostaglandins are important mediators in many inflammatory diseases including osteoporosis [45], the extent of metabolism of genistein to 4-ethylphenol may be clinically relevant.

We did not detect 1,3,5-trihydroxybenzoic acid or 1,3,5-trihydroxybenzene in any biological sample. Coldham *et al* (2002) also failed to detect either of these theoretically possible genistein metabolites following gut microfloral metabolism of

genistein [24]. It is therefore likely that these proposed genistein metabolites either do not form *in vivo* or are rapidly subjected to further metabolism.

The extent of disappearance of genistein from the digestive tract prior to the terminal ileum was considerably greater than that of daidzein. This may be a result of differences in the sites of metabolism and absorption of the two isoflavones in the intestine. Both genistein and daidzein are absorbed to some degree directly from the stomach in rats [46], however differences exist in the sites of intestinal absorption of the two isoflavones. Previous studies which attempted to examine isoflavone absorption by measuring uptake by cell lines derived from various regions of the intestine have perhaps been misleading. Although genistein uptake has been demonstrated in the colonic Caco-2 cell line [47], genistein absorption does not occur in intact colon tissue [48]. Although genistein is metabolised throughout the small and large intestines, absorption of unmetabolised genistein is limited to the jejunum and ileum [48]. The intestinal sites at which absorption of genistein metabolites occurs are unknown. Daidzein metabolism appears to be limited to sites in which gut microflora reside, namely the distal small intestine and colon. Absorption of unmetabolised daidzein occurs by passive transport and is greatest in the distal rather than the proximal or medial small intestine [49] however whether daidzein or its metabolites are also absorbed from the colon remains to be clarified. In the present study very little dietary genistein was detected at the terminal ileum, however a relatively large proportion of dietary daidzein was present in ileal digesta and therefore entered the colon. Whether or not daidzein and/or its metabolites, particularly equol, are absorbed from the colon needs to be determined as a lack of colonic absorption of daidzein or equol would impact considerably on estimates of daidzein bioavailability. In the present study considerable post-ileal disappearance of daidzein was observed, however it is not possible to ascertain if this disappearance was a result of absorption of daidzein and equol or further metabolism of daidzein and equol to other, as yet unknown, molecules.

For both genistein and daidzein, mean 24-hour genistein or daidzein input (dietary intake) exceeded mean 24-hour output (excretion of the isoflavone and its metabolites) by a substantial amount. This result indicates that either substantial amounts of the isoflavones or their metabolites accumulate in body tissues, as yet

unidentified metabolites of both daidzein and genistein exist which are excreted in urine and faeces or that the known genistein and daidzein metabolites are further catabolised. Isoflavones do not appear to accumulate substantially in body tissues, as a study using radio-labelled genistein found that only 0.6% of ingested genistein was incorporated into body tissues in female rats [50]. It is therefore likely that the apparent imbalance between isoflavone intake and excretion in the present study was due to the existence of unidentified daidzein and genistein metabolites and/or that the known daidzein and genistein metabolites are further broken down by either endogenous mammalian or gut microflora enzymes.

Phytoestrogens such as genistein and daidzein are best known for their ability to mimic the action of mammalian oestrogens. Plasma concentrations of genistein, daidzein and equol in the present study were similar to those reported in other studies [51, 52]. At a point in time following 4-weeks of genistein or daidzein supplementation, estimated total plasma quantities of isoflavone-derived oestrogenic compounds, (daidzein, equol and genistein) were approximately 0.2nM. In comparison, the total plasma quantity of 17β-estradiol in non-ovariectomised female rats is approximately one hundred times lower [53]. Although oestrogenic activity of phytoestrogens is less than that of mammalian oestrogens [54], the quantities of the oestrogenic isoflavones in plasma observed in the present study could be expected to be of physiological significance. In addition to their oestrogenic activity, isoflavones also have non-oestrogenic bioactivity [55-57]. As previously mentioned, 4-ethylphenol is bioactive [26] and the presence of this metabolite in plasma may also contribute to the physiological effects of genistein.

There was no evidence that inclusion of DHA in the diet altered daidzein or genistein metabolism, flow through the digestive tract or concentration in either plasma or urine. As many factors influence isoflavone metabolism and bioavailability it is important to quantify isoflavone absorption and the extent of metabolite formation in epidemiological and intervention studies aimed at determining a health benefit of isoflavones.

Overall, the present study has demonstrated that there is a very substantial disappearance of genistein in the upper digestive tract of the ovariectomised rat given

a genistein-supplemented diet. This observation coupled with the detection of genistein and its metabolites in plasma and urine demonstrates that the genistein given to the animals was bioavailable, although the extent of the bioavailability was unable to be determined with accuracy. Plasma and urinary contents suggest that quantitatively significant amounts of genistein or its metabolites are absorbed in the ovariectomised rat. The disappearance of daidzein to the end of the small intestine and over the total digestive tract was much lower than for genistein, though a considerable amount of daidzein plus its metabolites did disappear in passage through the tract. The detection of daidzein and equol in plasma and urine in significant quantities also indicates that this bioactive was absorbed but to an unknown degree.

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CHAPTER 5

Interaction between docosahexaenoic acid and oestrogens: Impact on bone mass post-ovariectomy in rats

In Chapter 2, DHA was found to have a greater protective effect than EPA or GLA on bone mass post-ovariectomy. As a possible beneficial effect of combined oestrogenic compound and long chain polyunsaturated fatty acid treatment has previously been reported, the aim of the study presented in this chapter was to determine if combined DHA and oestrogenic compound treatment would have an additive or synergistic effect on bone mass post-ovariectomy.

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Abstract

A possible synergistic effect of 17β-oestradiol treatment and dietary supplementation with long chain polyunsaturated fatty acids (LCPUFAs) in minimising post-ovariectomy bone loss in rats has previously been reported. Similarly, a beneficial effect of dietary supplementation with both n-3 LCPUFAs and phytoestrogens on bone mass post-ovariectomy has also been proposed. With the recent finding that the specific n-3 LCPUFA docosahexaenoic acid (DHA, 22:6n-3) is particularly bone-protective we sought to determine whether there was an additive or synergistic effect of treatment with DHA and oestrogenic compounds (17β-oestradiol or either of the phytoestrogens, genistein or daidzein) on bone mass in ovariectomised (OVX) rats.

One hundred and four, six-month old Sprague-Dawley rats were randomised into 9 groups and either ovariectomised (8 groups) or sham operated (1 group, n=10). Using a 2×4 factorial design approach, OVX animals received DHA (0.5g/kg body weight/day) or no DHA and either no oestrogenic compound, genistein (20mg/kg body weight/day), daidzein, (20mg/kg body weight/day) or 17β-oestradiol (1μg/day). Sham-operated animals received no DHA and no oestrogenic compound. Study duration was 18 weeks. Main outcome measures were bone mineral content (BMC), bone area (BA) and bone mineral density (BMD) measured by DEXA and pQCT. Plasma concentration of carboxylated and under-carboxylated osteocalcin, IL-6 and red blood cell (RBC) fatty acid composition were determined at study completion. Femur (F) BMC was significantly higher in animals treated with DHA or 17β-oestradiol compared to untreated ovariectomised controls. DHA treatment was associated with significantly higher plasma concentration of carboxylated osteocalcin compared to untreated ovariectomised controls (p=0.02). Plasma concentration of total osteocalcin was significantly lower in 17β-oestradiol treated animals compared to untreated ovariectomised controls (p=0.01). There were significant interactions between oestrogenic compound and DHA treatment for F BMC (p=0.02), plasma IL-6 concentration (p=0.03) and the percentages of various fatty acids within RBCs. The Final F BMC was significantly greater in animals treated with DHA and 17β-oestradiol compared to either treatment alone (p=0.01). No beneficial effect of combined DHA and genistein or daidzein treatment on bone mass was apparent. Plasma Il-6 concentration was significantly lower in animals treated with 17β-oestradiol and DHA compared to 17β-oestradiol alone (p=0.01). The percentage of

n-3 LCPUFAs in RBCs was significantly greater in animals treated with 17β-oestradiol and DHA compared to either treatment alone. In contrast the ratio of n-3:n-6 LCPUFAs in RBCs was significantly lower in animals treated with daidzein and DHA compared to DHA treatment alone.

Introduction

Oestrogen decline due to natural or surgical menopause results in loss of bone mass and can lead to the development of osteoporosis. One of the consequences of oestrogen deficiency is disruption of long chain polyunsaturated fatty acid (LCPUFA) metabolism such that tissue and blood concentrations of very long chain PUFAs decline [1, 2]. Dietary supplementation with n-3 LCPUFAs increases tissue and blood levels of n-3 LCPUFAs and assists in maintaining bone mass post-ovariectomy in rodents [3] and post-menopause in women [4]. Oestrogen Replacement Therapy (ERT or HRT) also increases plasma concentrations of the very long chain n-3 PUFAs, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) in post-menopausal women and this is believed to contribute to the observed anti-atherosclerotic effects of ERT [5]. In 1999, Schlemmer *et al* reported a possible synergistic effect of 17β-oestradiol therapy in conjunction with dietary supplementation with gamma-linolenic acid (GLA, 18:3n-6) and EPA in ameliorating ovariectomy-induced bone mineral loss in rats [6]. One other study has reported a possible reduction in rate of bone resorption in ovariectomised rats receiving a soy-containing diet supplemented with menhaden oil (rich in EPA and DHA) [7]. Since this time, the relative effectiveness of GLA, EPA and DHA in preventing ovariectomy-induced bone mineral loss in rats has been examined and DHA has been identified as having the strongest bone-sparing effect [Chapter 2]. The aim of the present study was to compare the effect on bone mass of dietary DHA supplementation with and without 17β-oestradiol treatment or phytoestrogen supplementation in the ovariectomised rat.

Method

Animals

One hundred and four female Sprague-Dawley rats were obtained from the Small Animal Production Unit, Massey University. At age 7 months (week 0 of the study),

animals were randomly assigned to one of nine groups and either ovariectomised (OVX) (1 group, n=10; 7 groups, n=12) or sham operated (1 group, n=10). The operations were performed under general anaesthetic (isofluorane). Sham operated animals were anaesthetised and an incision made but the ovaries left intact. The ovaries were removed from the OVX animals. The ovariectomised animals were allocated to one of 8 treatment groups based on a 4 x 2 factorial design. Treatments consisted of an oestrogenic compound (genistein (GEN), daidzein (DAI), 17β-oestradiol (OES) or none) with or without DHA as shown in **Table 1**.

Table 1 *Allocation of treatments to study groups*

Group Name	Operation	Oestrogenic Compound	DHA
SHAM	Sham	None	No
OVX	Ovariectomy	None	No
DHA	Ovariectomy	None	Yes
GEN	Ovariectomy	Genistein	No
GENDHA	Ovariectomy	Genistein	Yes
DAI	Ovariectomy	Daidzein	No
DAIDHA	Ovariectomy	Daidzein	Yes
OES	Ovariectomy	17β-oestradiol	No
OESDHA	Ovariectomy	17β-oestradiol	Yes

At time of surgery, a 90-day slow-release 17β-oestradiol pellet (providing 1μg 17β-oestradiol/day, NE-121 Innovative Research of America, USA) was inserted under the skin at the nape of the neck in two groups of ovariectomised animals ("OES" and "OESDHA", n=12). All other animals received a placebo pellet (NC-111 Innovative Research of America, USA) inserted in the same way. Sixty-day release pellets were inserted in the OES and OESDHA animals at study week 12 to ensure continual oestradiol/placebo dosing throughout the remainder of the trial.

All animals were maintained in individual shoebox cages at 22°C (± 2°C) with a 12h/12h light/dark cycle in a dedicated room in the Small Animal Production Unit at Massey University. All animals had *ad libitum* access to deionised water. The study was approved by the Massey University Animal Ethics Committee (Approval number 03/102).

Diets

Animals were acclimated to a nutritionally balanced, semi-synthetic diet containing 14% caseinate, 5% cellulose, 4% corn oil, 1.25% calcium carbonate (providing 0.5% calcium), 60% starch with added vitamins and minerals formulated based on AIN93M, for four weeks prior to ovariectomy (week -4 to week 0). The sham operated "SHAM", (n = 10), ovariectomised control "OVX" (no oestrogenic compound, no DHA n=10) and the oestrogen control "OES" (17β-oestradiol, no DHA, n=12) groups were maintained on this diet for the 18 week study period. Of the remaining six ovariectomised experimental groups two groups were fed the base diet with added genistein (20mg/kg body weight/day) "GEN" (genistein, no DHA n=12) and "GENDHA" (genistein +DHA, n-12) and two with added daidzein (20mg/kg body weight/day) "DAI" (daidzein, no DHA, n=12) and "DAIDHA" (daidzein + DHA, n=12). The GENDHA, DAIDHA and the remaining two ovariectomised experimental groups: "DHA" (no oestrogenic compound + DHA, n=12) and "OESDHA" (17β-oestradiol + DHA, n=12) received 0.5g docosahexaenoic acid ethyl ester/kg mean body weight/day. Genistein (95%) and daidzein (96%) were supplied by LC Laboratories, PKC Pharmaceuticals, Inc, Woburn MA, USA. DHA ethyl ester (80%, 01177B-E80 was supplied by Sanmark LLC, USA. Diet compositions are shown in **Table 2**.

The animals were weighed weekly, and the amount of phytoestrogen and DHA added to each test diet was adjusted according to the mean body weight of animals in each treatment group. In groups receiving DHA, the amount of corn oil in the diet was reduced so the oil content of all diets totalled 4%. All diets contained at least 2% corn oil, an amount in excess of the minimum amount required to prevent n-6 EFA deficiency (1%). Diets were randomly sampled and analysed in order to confirm nutrient quantity.

Sham operated animals were fed *ad libitum.* The food intake of ovariectomised animals was limited to that of the sham controls in order to reduce ovariectomy-induced weight gain.

Table 2 *Ingredient composition (% air-dry weight) of control and experimental diets.*

	SHAM	OVX	Dai	Gen	Oes	DHA	Dai+ DHA	Gen+ DHA	Oes+ DHA
Cornstarch	59.7	59.7	59.674	59.674	59.7	59.7	59.674	59.674	59.7
Sodium Caseinate	14	14	14	14	14	14	14	14	14
Sucrose	6	6	6	6	6	6	6	6	6
Cellulose	5	5	5	5	5	5	5	5	5
Vitamins[a]	5	5	5	5	5	5	5	5	5
Minerals (excl. Ca[b])	5	5	5	5	5	5	5	5	5
Calcium Carbonate	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3	1.3
Corn Oil[c,d]	4	4	4	4	4	3.3	3.3	3.3	3.3
DHA ethyl ester[c]	0	0	0	0	0	0.7	0.7	0.7	0.7
Genistein	0	0	0	0.026	0	0	0	0.026	0
Daidzein	0	0	0.026	0	0	0	0.026	0	0

[a] Supplying (mg/kg diet) retinol acetate 5.0, $_{DL}$-α-tocopherol acetate 200.0, menadione 3.0, thiamine hydrochloride 5.0, riboflavin 7.0, pyridoxine hydrochloride 8.0, D-pantothenic acid 20.0, folic acid 2.0, nicotinic acid 20.0, D-biotin 1.0, myo-inositol 200.0, choline chloride 1500; (μg/kg diet) ergocalciferol 25.0, cyanocobalamin 50.0.

[b] Supplying (g/kg diet) chloride 7.79, magnesium 1.06, phosphate 4.86, potassium 5.24, sodium 1.97; (mg/kg diet) chromium 1.97, copper 10.7, iron 424, manganese 78.0, zinc 48.2; (μg/kg diet) cobalt 29.0, iodine 105.0, molybdenum 152.0, Selenium 151.0

[c] DHA dose was 0.5g/kg mean rat body weight/day. The percentages of DHA and corn oil in the diet shown in this table are the percentages at trial commencement. The percentage of DHA in diets increased and percentage of corn oil decreased as the body weight of animals increased over the trial period. At trial completion the percentage of DHA in the diet was 0.9% and the percentage of corn oil was 3.1%.

[d] Fatty acid composition of corn oil: 58% linoleic acid, 28% oleic acid, 11% palmitic acid and 2% stearic acid.

DHA = Docosahexaenoic Acid (22:6n-3)

Dual Energy X-Ray Absorptiometry (DEXA) Scans

Bone mineral contents and densities were determined with a Hologic QDR4000 bone densitometer using a pencil beam unit (Bedford, USA). Prior to scanning animals were anaesthetised with a mixture of 0.2ml Acepromazine (ACP) + 0.5ml Ketamine + 0.1mL Xylazine + 0.2ml sterile water, at a dosage of 0.05ml/100g body weight administered intra-peritoneally via a 25G x 15.875 mm needle. A suitable level of anaesthesia was attained after five to ten minutes and was maintained for up to 2 hours. Anaesthetised rats were placed in a supine position on an acrylic platform of uniform 38.1mm thickness so that the femur was at right angles to the long axis of the spine and similarly, at right angles to the tibia ("frog-leg" position). Regional high-resolution scans of both femurs and the lumbar spine were performed using a 1.524 mm diameter collimator with 0.30516 mm point resolution and 0.64516 mm

line spacing. Scans were made at weeks –2 (baseline), 5 and 18. A daily quality control (QC) scan was made to ensure precision met with the required coefficient of variation.

Blood Sampling

At week 8, rats were placed in a purpose-built restrainer, which was then placed on top of a heat pad under a heat lamp. A single blood sample of approximately 1ml was withdrawn from the lateral tail vein, using a 23G x 15.875 mm hypodermic needle and 1ml syringe. Blood samples were collected into vacutainers containing heparin and centrifuged at 3000 rpm for 10 minutes. The plasma was removed, snap-frozen with liquid nitrogen, and stored at –80 °C for later analysis of 17β-oestradiol.

Euthanasia and Dissection

At week 18 after final DEXA scans, animals were anaesthetized. The animals were subsequently exsanguinated by cardiac puncture with a 19G x 38.1mm needle and 5ml syringe. Two blood samples were collected from each animal, one into a vacutainer containing heparin and one into a vacutainer containing EDTA. Samples were centrifuged at 3000 rpm at 4°C for 10 minutes. Plasma was collected from heparinised samples and stored at –80 °C for later analysis. Plasma was discarded from samples in EDTA and the remaining red blood cells washed with isotonic saline and stored at –80 °C pending analysis of fatty acid composition.

The uteri and adnexae were removed and their wet weight determined as a quality control measure to confirm success of ovariectomy. Both rear legs and the spine were removed, and stored in phosphate buffered saline at -20°C pending biomechanical testing and CT analysis.

Red Blood Cell Fatty Acid Composition

Red blood cell fatty acid composition was determined by direct transmethylation followed by gas chromatography. To each sample, 1ml of internal standard (2.5mg/ml tricosanoic acid methyl ester (C23:0, Sigma-Aldrich Chemicals) dissolved in chloroform), 2ml of toluene and 5ml of 5% sulphuric acid in methanol was added. Tubes were sealed, shaken and fatty acid methyl esters (FAMEs) formed by heating

at 80°C for 1 hour. Samples were then cooled and shaken with 5ml of saturated NaCl, then centrifuged at 2000RCF for 10 minutes at 10°C. The upper toluene layer was collected and 1µl (with 1:100 split) injected into an Agilent 6890 Gas Chromatograph with auto sampler and FID detector. A SGHE Sol Gel Wax column was used with a column length of 30 metres, internal diameter 0.25mm and film thickness 0.25µm. Hydrogen flow rate was 1.5ml/min, constant flow and average linear velocity was 50cm/sec. The initial injection temperature was 170°C and temperature was ramped at 1°C/minute to 225°C. Fatty acids in the samples were determined by comparison with known standards supplied by Sigma-Aldrich Chemicals (37-component FAME mixture C4:0 – C24:0, PUFA 1 and PUFA 3) and Restek Bellefonte, PA, USA (NLEA FAME Mix, 28 components).

Plasma concentrations of 17β-oestradiol, IL-6 and osteocalcin

Concentration of 17β-oestradiol in rat plasma was determined at week 8 post-ovariectomy using a commercially available RIA kit purchased from Diasorin, Saluggia, Italy. Plasma concentrations of IL-6, osteocalcin and under-carboxylated osteocalcin were determined at trial completion using commercially available ELISA kits as follows: Quantikine Rat IL-6 Immunoassay ELISA kit (Cat.# SR6000B, R&D Systems, Minneapolis, USA), Rat Glu-Oc (Cat.# MK122) and Rat Gla-Oc (Cat.# MK121) competitive EIA Kits (Takara Bio Inc., Otsu, Shiga, Japan).

Computed Tomography (CT)

Following completion of the trial, the opportunity arose to access a pQCT scanner. As both femurs had previously been subjected to destructive testing (biomechanical testing and bone marrow fatty acid analysis), the right tibia was used for pQCT analysis. After removal of skin and disarticulation, tibia length was determined manually with callipers, and the tibia was positioned for scanning on a plastic cradle. Scans were made with a XCT2000 pQCT scanner (Stratec, Pforzheim, Germany) 5mm from the proximal end of the tibia (at a constant site in the proximal metaphysis) and at 50% of the length of the tibia (mid-diaphysis). Voxel size was 0.1mm and scan speed was 5mm per second. Scans were analysed using the manufacturer's software; the contour threshold was 280mg/cm^3 and SSI threshold was 540mg/cm^3. Main outcome measures were trabecular BMC and BMD

(determined in the 5mm slice) and cortical BMC, BA and BMD, and endosteal and periosteal circumferences (determined at 50% of tibia length).

Biomechanical Testing

Right femurs were scraped free of adhering flesh and maintained in PBS at room temperature for 1 hour prior to testing. The length of each femur was measured with an electronic calliper and the midpoint marked. Both the anterior-posterior and latero-medial diameter at the midpoint of the femur were similarly determined.

The maximum load, breaking load, maximum deformity (stroke length), breaking stress, breaking strain, (the percent deformation of the femur just prior to the time of breaking) the breaking energy (the amount of energy required to break the femur) and elastic modulus (force required to bend the bone in the elastic phase of deformation) were determined using a Shimadzu Ezi-test (Kyoto, Japan) materials testing machine. The femurs were subjected to a three point bending test with the application point of the upper fulcrum positioned midway between the two supporting rods of the testing jig; the supporting rods were 15mm apart. Load was applied at a constant deformation rate of 50mm/min at the midpoint of the anterior surface of the femur.

Statistical Analysis

Bone densitometry data were analysed by repeated measures mixed model analysis (Proc Mixed) using SAS 9.1® (SAS Institute Incorporated, Cary, UC, USA). In all cases the Toeplitz model was found to give the best fit to the data. All other data conformed to the requirements of the general linear model and were analysed by two-way ANOVA (Proc GLM) with factorial-design analysis using SAS 9.1® (SAS Institute Incorporated, Cary, UC, USA). For the overall ANOVA, $p<0.05$ was considered statistically significant.

Selected contrasts of interest were included in the original ANOVA in keeping with the available degrees of freedom. Main effects, (i.e. the effects of treatment with daidzein, genistein, 17β-oestradiol and DHA) were contrasted with untreated ovariectomy. The effects of combined treatment with DHA and each oestrogenic compound were contrasted with the effect of treatment with either compound alone (ie contrasts were made as follows: OESDHA vs DHA, OESDHA vs OES,

GENDHA vs DHA, GENDHA vs GEN, DAIDHA vs DHA, DAIDHA vs DAI.) A Bonferroni correction was made to protect the overall error rate and as a result $p<0.01$ was considered statistically significant for all contrasts.

Results

Diets

The formulated nutrient compositions of the diets were confirmed by the conduct of proximate analysis on random diet samples (data not shown). The digestibility, metabolism and bioavailability of the genistein and daidzein supplements used were assessed in a separate study [Chapter 4] involving ovariectomised rats of the same age as used in the present study. In brief, mean plasma concentrations of genistein, daidzein and equol for the same dosage rate as used in the present study were 24 nmol/L, 17 nmol/L and 18 nmol/L respectively. Inclusion of DHA in the diet did not affect genistein or daidzein metabolism, digestibility or bioavailability [Chapter 4].

Animal body weights and food intake

Baseline animal body weights were not significantly different between groups (mean weight 313.5 ± 2.93g). Final rat body weight was significantly higher in ovariectomised compared to sham-operated animals ($p=0.0003$) (**Table 3**). Dietary DHA supplementation had no statistically significant effect on either average daily food intake or final rat body weight. There was no statistically significant effect of any oestrogenic compound on mean daily food intake. However, treatment with 17β-oestradiol or daidzein was associated with significantly lower final rat body weight compared to no oestrogenic treatment or treatment with genistein ($p=0.0005$ and $p=0.0015$ respectively). There was no statistically significant effect of combined treatment with DHA and any of the three oestrogenic compounds on mean daily food intake or final animal body weight.

Table 3 *Mean daily food intake, final (week 18) body weight and plasma concentration of 17β-oestradiol measured at week 8*

	Sham	**Ovariectomised**								***P-values***			
	No Oest. No DHA	**No Oest. No DHA**	**Oest. Cmpd**			**No Oest.**	**Combined treatment**				***Effect of ovx***	***Effect of treatment***		
	Sham	**OVX**	**Dai**	**Gen**	**Oes**	**DHA**	**Dai+ DHA**	**Gen+ DHA**	**Oes+ DHA**	**SE**	***Ovx***	***Oest. Cmpd***	***DHA***	***Oest. Cmpd × DHA***
Mean daily food intake[#] (g)	18.3	18.4	18.0	18.8	17.4	18.9	18.0	18.9	18.2	0.52	*0.92*	*0.18*	*0.35*	*0.81*
Final rat body weight (g)	341.9	408.0	363.3*	410.5	363.9*	412.4	378.3	399.8	369.8	12.8	*0.002*	*0.0009*	*0.69*	*0.80*
17β-oestradiol (pmol/L)	77.5	BD	BD	BD	40.3	BD	BD	BD	42.3	6.7	*<0.0001*	*<0.0001*	*0.55*	*0.93*

Female rats were ovariectomised (ovx) or sham operated and treated with either daidzein (DAI, 20mg/kg body weight/day), genistein (GEN, 20mg/kg body weight/day), 17β-oestradiol (OES, 1μg/day) or docosahexaenoic acid (DHA, 0.5g/kg body weight/day) or a combination of oestrogenic compound and DHA for 18 weeks.
BD Below detectable limits of assay
[#] Food intake of ovariectomised animals was limited to that of sham controls to minimise ovariectomy-induced weight gain.

Main Effects Contrasts*: The effect of each of the three oestrogenic compounds was contrasted with the effect of untreated ovariectomy (OVX). The effect of DHA treatment was contrasted with the effect of untreated ovariectomy.*
* *Significantly different from OVX at p<0.01 (Contrast made for main effects only)*

Combined Treatment Contrasts: *Contrasts were constructed for each combined treatment of oestrogenic compound +DHA compared to treatment with the oestrogenic compound alone and DHA alone. Differences between combined treatments and the component treatments alone were not statistically significant at p<0.01.*

Table 4 *Percentage of long chain polyunsaturated fatty acids in total red blood cell lipids*

	Sham	Ovariectomised									P-values			
	No Oest. No DHA	No Oest. No DHA	Oest. cmpd			No Oest.	Combined treatment				*Effect of ovx*	*Effect of treatment*		
	Sham	OVX	Dai	Gen	Oes	DHA	Dai+ DHA	Gen+ DHA	Oes+ DHA	SE	*Ovx*	*Oest. Cmpd*	*DHA*	*Oest. Cmpd × DHA*
AA	21.3	19.8	20.7	20.4	21.6*	16.4*	$18.3^{A,B}$	16.4^{B}	$18.4^{A,B}$	0.39	*0.06*	*0.03*	*<0.0001*	*0.05*
EPA	0.49	0.54	0.45	0.44	0.67*	1.04*	0.91^{B}	0.90^{B}	$1.36^{A,B}$	0.04	*0.99*	*0.0008*	*<0.0001*	*0.0009*
DHA	5.8	5.5	5.4	5.4	6.1	7.7*	7.5^{B}	7.5^{B}	$8.3^{A,B}$	0.22	*0.02*	*0.0001*	*<0.0001*	*0.70*
AA:DHA	6.2	6.4	7.1*	7.7*	6.5	2.1*	2.4^{B}	2.1^{B}	2.1^{B}	0.24	*0.001*	*0.0006*	*0.001*	*0.002*
EPA:AA	0.03	0.03	0.03	0.03	0.04	0.06*	$0.05^{A,B}$	0.06^{B}	$0.08^{A,B}$	0.004	*0.66*	*0.02*	*<0.0001*	*0.03*
EPA:DHA	0.06	0.07	0.06	0.06	0.08	0.13*	0.12^{B}	0.12^{B}	$0.16^{A,B}$	0.007	*0.66*	*0.03*	*<0.0001*	*0.02*
Total n-3	6.8	6.6	6.3	6.3	7.4*	9.6*	9.2^{B}	9.1^{B}	$10.6^{A,B}$	0.30	*0.13*	*<0.0001*	*<0.0001*	*0.09*
Total n-6	21.7	20.3	21.1	20.9	22.0*	17.9*	$18.9^{A,B}$	16.9^{B}	$18.9^{A,B}$	0.38	*0.56*	*<0.0001*	*0.009*	*0.04*
Ratio n-3:n-6	0.35	0.35	0.32	0.35	0.37	0.56*	$0.50^{A,B}$	0.57^{B}	0.58^{B}	0.02	*0.46*	*0.006*	*<0.0001*	*0.08*

Female rats were ovariectomised (ovx) or sham- operated and treated with either daidzein (DAI, 20mg/kg body weight/day), genistein (GEN, 20mg/kg body weight/day), 17β-oestradiol (OES, 1μg/day) or docosahexaenoic acid (DHA, 0.5g/kg body weight/day) or a combination of oestrogenic compound and DHA for 18 weeks. Results are expressed as the mean percentage for each study group.

Main Effects Contrasts*: The effect of each of the three oestrogenic compounds was contrasted with the effect of untreated ovariectomy (OVX). The effect of DHA treatment was contrasted with the effect of untreated ovariectomy.*

* *Significantly different from OVX at p<0.01(Contrast made for main effects only)*

Combined Treatment Contrasts: *Contrasts were constructed for each combined treatment of oestrogenic compound + DHA compared to treatment with the oestrogenic compound alone and DHA alone.*

[A] *Significantly different from DHA alone at p<0.01 (Contrast made for combined treatment only)*

[B] *Significantly different from oestrogenic compound alone at p<0.01 (Contrast made for combined treatment only)*

Red Blood Cell Fatty Acid Composition

The ratio of AA relative to DHA in RBCs was significantly higher in ovariectomised animals compared to sham controls ($p=0.001$, **Table 4**). Treatment with genistein or daidzein was associated with a significantly higher ratio of AA relative to DHA in RBCs compared to untreated ovariectomy ($p<0.0001$). In contrast, DHA supplementation was associated with a significantly lower ratio of AA relative to DHA in RBCs compared to untreated ovariectomy ($p<0.0001$). The proportion of EPA in RBCs was also significantly influenced by DHA and oestrogenic treatment and there was a statistically significant interaction between the two treatments for the percentage of EPA in RBCs ($p=0.0009$). In the absence of oestrogenic compounds, DHA treatment was associated with a significantly higher percentage of EPA in RBCs compared to untreated ovariectomy ($p<0.0001$). The proportion of EPA in RBCs was greater in animals treated with 17β-oestradiol and DHA compared to either DHA or 17β-oestradiol treatment alone ($p<0.0001$). Similarly, the proportion of DHA in RBCs was also significantly higher in animals treated with both 17β-oestradiol and DHA compared to either treatment alone ($p<0.0001$). Overall, the percentage of both n-3 and n-6 LCPUFAs was significantly higher, and the ratio of AA relative to DHA significantly lower, in animals treated with both 17β-oestradiol and DHA compared to animals receiving DHA alone ($p<0.0001$). In contrast, the percentage of n-6 LCPUFAs in RBCs was significantly higher in animals treated with a combination of daidzein and DHA compared to DHA alone ($p=0.01$).

Bone Densitometry – Lumbar Spine & Femur

Ovariectomy resulted in significantly lower LS BMC and BMD at week 18 (**Table 5**), and F BMC and BMD at weeks 5 and 18, compared to sham (**Table 6**).

Over time, LS BMC was significantly influenced by treatment with oestrogenic compounds and DHA ($p<0.0001$ and $p=0.04$ respectively) and there was a significant interaction between the two treatments ($p=0.04$). Final LS BMC was significantly higher in animals treated with 17β-oestradiol than untreated ovariectomy ($p=0.0002$). However final LS BMC in animals treated with DHA was not significantly greater than untreated ovariectomy ($p=0.07$). Similarly, final LS BMC in animals treated with both 17β-oestradiol and DHA was not significantly different from animals treated with 17β-oestradiol alone ($p=0.07$). Final LS BMC in animals treated with

both daidzein and DHA was lower than in animals treated with DHA alone ($p=0.04$) although the difference failed to reach the level of significance required ($p<0.01$) for the contrast following Bonferroni correction. There was a significant interaction between oestrogenic compound and DHA on LS BA ($p=0.004$). Final LS BA in animals treated with both daidzein and DHA was significantly lower than for animals treated with DHA alone ($p=0.01$). LS BMD was significantly greater in animals treated with 17β-oestradiol compared to untreated ovariectomy at both week 5 ($p=0.007$) and week 18 post-surgery ($p<0.0001$) (**Table 5**).

At week 5, F BMC in animals treated with genistein, 17β-oestradiol or DHA was significantly greater than in untreated ovariectomised animals ($p=0.01$, $p=0.0006$ and $p=0.01$ respectively). However at week 18, F BMC was greater than untreated ovariectomised animals only for animals receiving the 17β-oestradiol or DHA treatments ($p<0.0001$ and $p=0.01$ respectively). There was a statistically significant interaction between treatment with oestrogenic compounds and DHA ($p=0.02$) and week 18 F BMC was significantly greater in animals treated with 17β-oestradiol and DHA compared to either 17β-oestradiol ($p<0.0001$) or DHA alone ($p=0.01$). Final F BMD was significantly greater in animals treated with 17β-oestradiol compared to untreated ovariectomy ($p<0.0001$) (**Table 6**).

Table 5 *Lumbar spine bone mineral content (BMC), bone area (BA) and bone mineral density (BMD) as measured by DEXA*

BMC (g)	**Sham**	**Ovariectomised**									**Repeated Measures ANOVA**			
	No Oest. No DHA	**No Oest. No DHA**	**Oest. Cmpd No DHA**			**No Oest.**	**Combined treatment**							
Time	**Sham**	**OVX**	**Dai**	**Gen**	**Oes**	**DHA**	**Dai+ DHA**	**Gen+ DHA**	**Oes+ DHA**	SE	**Factor**	***p-value***	**Factor**	***p-value***
Week 0	0.53	0.51	0.51	0.51	0.54	0.51	0.50	0.50	0.52	0.02	Time	<0.0001	Oest.×DHA	*0.04*
Week 5	0.56	0.48	0.51	0.50	0.57	0.52	0.50	0.49	0.58	0.02	Ovx	0.0001	Time×Ovx	*0.04*
Week 18	0.56*	0.47	0.49	0.50	0.56*	0.52	0.48	0.50	0.59[A]	0.02	Oest.	<0.0001	Time×Oest	*0.21*
											DHA	0.04	Time×DHA	*0.18*
													Time×Oest.×DHA	*0.85*
BA (cm^2)	**Sham**	**Ovariectomised**									**Repeated Measures ANOVA**			
	No Oest. No DHA	**No Oest. No DHA**	**Oest. Cmpd No DHA**			**No Oest.**	**Combined treatment**							
Time	**Sham**	**OVX**	**Dai**	**Gen**	**Oes**	**DHA**	**Dai+ DHA**	**Gen+ DHA**	**Oes+ DHA**	SE	**Factor**	***p-value***	**Factor**	***p-value***
Week 0	2.02	2.00	2.00	2.00	2.04	2.00	1.99	1.98	2.02	0.04	Time	*<0.0001*	Oest.×DHA	*0.004*
Week 5	2.06	2.02	2.04	2.04	2.05	2.04	2.02	2.02	2.07	0.03	Ovx	*0.10*	Time×Ovx	*0.85*
Week 18	2.08	2.06	2.03	2.08	2.09	2.09	2.03[A]	2.07	2.12	0.04	Oest.	*0.07*	Time×Oest.	*0.23*
											DHA	0.07	Time×DHA	*0.28*
													Time×Oest.×DHA	*0.93*
BMD (g/cm^2)	**Sham**	**Ovariectomised**									**Repeated Measures ANOVA**			
	No Oest. No DHA	**No Oest. No DHA**	**Oest. Cmpd No DHA**			**No Oest.**	**Combined treatment**							
Time	**Sham**	**OVX**	**Dai**	**Gen**	**Oes**	**DHA**	**Dai+ DHA**	**Gen+ DHA**	**Oes+ DHA**	SE	**Factor**	***p-value***	**Factor**	***p-value***
Week 0	0.26	0.25	0.26	0.26	0.26	0.26	0.25	0.25	0.26	0.006	Time	<0.0001	Oest.×DHA	*0.25*
Week 5	0.27*	0.24	0.25	0.24	0.28*	0.26	0.25	0.24	0.28[A]	0.006	Ovx	<0.0001	Time×Ovx	*0.0001*
Week 18	0.27*	0.23	0.24	0.24	0.27*	0.25	0.24	0.24	0.28[A]	0.007	Oest.	<0.0001	Time×Oest.	*0.02*
											DHA	0.07	Time×DHA	*0.25*
													Time×Oest.×DHA	*0.84*

Female rats were ovariectomised (ovx) or sham- operated and treated with daidzein (DAI, 20mg/kg body weight/day), genistein (GEN, 20mg/kg body weight/day), 17β-oestradiol (OES, 1μg/day) or docosahexaenoic acid (DHA, 0.5g/kg body weight/day) or a combination of oestrogenic compound and DHA for 18 weeks. Results are expressed as the mean for each study group.

Main Effects Contrasts*: The effect of each of the three oestrogenic compounds was contrasted with the effect of untreated ovariectomy (OVX). The effect of DHA treatment was contrasted with the effect of untreated ovariectomy.*

* *Significantly different from OVX at p<0.01(Contrast made for main effects and ovariectomy vs sham only)*

Combined Treatment Contrasts: *Contrasts were constructed for each combined treatment of oestrogenic compound +DHA compared to treatment with the oestrogenic compound alone and DHA alone.*

[A] *Significantly different from DHA alone at p<0.01(Contrast made for combined treatment only)*

Table 6 *Femur bone mineral content (BMC), bone area (BA) and bone mineral density (BMD) as measured by DEXA*

BMC (g)	**Sham**	**Ovariectomised**									**Repeated Measures ANOVA**			
	No Oest. No DHA	**No Oest. No DHA**	**Oest. Cmpd No DHA**			**No Oest.**	**Combined treatment**				**Factor**	***p-value***	**Factor**	***p-value***
Time	**Sham**	**OVX**	**Dai**	**Gen**	**Oes**	**DHA**	**Dai+ DHA**	**Gen+ DHA**	**Oes+ DHA**	SE	Time	0.02	Oest.×DHA	*0.02*
											Ovx	0.0001	Time×Ovx	*0.21*
Week 0	0.55	0.54	0.54	0.54	0.55	0.55	0.54	0.54	0.56	0.01	Oest.	<0.0001	Time×Oest	*0.17*
Week 5	0.61*	0.51	0.54	0.55*	0.61*	0.56*	0.54	0.53	0.62[A]	0.009	DHA	0.01	Time×DHA	*0.79*
Week 18	0.58*	0.49	0.48	0.50	0.55*	0.51*	0.50	0.50	0.58[A,B]	0.01			Time×Oest.×DHA	0.93
BA (cm^2)	**Sham**	**Ovariectomised**									**Repeated Measures ANOVA**			
	No Oest. No DHA	**No Oest. No DHA**	**Oest. Cmpd No DHA**			**No Oest.**	**Combined treatment**				**Factor**	***p-value***	**Factor**	***p-value***
Time	**Sham**	**OVX**	**Dai**	**Gen**	**Oes**	**DHA**	**Dai+ DHA**	**Gen+ DHA**	**Oes+ DHA**	SE	Time	<0.0001	Oest.×DHA	*0.07*
											Ovx	0.45	Time×Ovx	*0.42*
Week 0	1.60	1.57	1.59	1.60	1.56	1.58	1.59	1.58	1.52	0.02	Oest.	0.07	Time×Oest.	*0.10*
Week 5	1.65	1.61	1.62	1.64	1.63	1.63	1.62	1.61	1.64	0.02	DHA	0.20	Time×DHA	*0.94*
Week 18	1.68	1.68	1.67	1.68	1.70	1.69	1.68	1.69	1.72	0.02			Time×Oest.×DHA	0.82
BMD (g/cm^2)	**Sham**	**Ovariectomised**									**Repeated Measures ANOVA**			
	No Oest. No DHA	**No Oest. No DHA**	**Oest. Cmpd No DHA**			**No Oest.**	**Combined treatment**				**Factor**	***p-value***	**Factor**	***p-value***
Time	**Sham**	**OVX**	**Dai**	**Gen**	**Oes**	**DHA**	**Dai+ DHA**	**Gen+ DHA**	**Oes+ DHA**	SE	Time	<0.0001	Oest.×DHA	*0.34*
											Ovx	<0.0001	Time×Ovx	*0.05*
Week 0	0.34	0.34	0.34	0.34	0.35	0.34	0.34	0.34	0.35	0.006	Oest.	<0.0001	Time×Oest.	*0.11*
Week 5	0.37*	0.32	0.33	0.33	0.37*	0.34	0.33	0.33	0.38[A]	0.005	DHA	0.02	Time×DHA	*0.78*
Week 18	0.37*	0.30	0.33	0.33	0.37*	0.34*	0.33	0.33	0.38[A]	0.004			Time×Oest.×DHA	0.98

Female rats were ovariectomised (ovx) or sham- operated and treated with daidzein (DAI, 20mg/kg body weight/day), genistein (GEN, 20mg/kg body weight/day), 17β-oestradiol (OES, 1μg/day) or docosahexaenoic acid (DHA, 0.5g/kg body weight/day) or a combination of oestrogenic compound and DHA for 18 weeks. Results are expressed as the mean for each study group.

Main Effects Contrasts*: The effect of each of the three oestrogenic compounds was contrasted with the effect of untreated ovariectomy (OVX). The effect of DHA treatment was contrasted with the effect of untreated ovariectomy.*

* *Significantly different from OVX at p<0.01(Contrast made for main effects and ovariectomy vs sham only)*

Combined Treatment Contrasts: *Contrasts were constructed for each combined treatment of oestrogenic compound +DHA compared to treatment with the oestrogenic compound alone and DHA alone.*

[A] *Significantly different from DHA alone at p<0.01* [B] *Significantly different from oestrogenic compound alone at p<0.01 (Contrasts made for combined treatment only)*

Trabecular and Cortical Bone Mineral Content, Area and Density

Ovariectomy was associated with significantly lower trabecular BMC and BMD compared to sham-operation ($p<0.0001$) (**Table 7**). Final trabecular BMC was significantly higher in DHA and 17β-oestradiol treated animals compared to untreated ovariectomised controls ($p=0.008$ and $p<0.0001$ respectively). Neither genistein nor daidzein treatment were associated with any significant effect on trabecular or cortical BMC, BMD or BA.

There was no evidence of an interaction between DHA supplementation and treatment with oestrogenic compounds on trabecular BMC, BMD or BA. Cortical BMC and BA tended to be higher in the group supplemented with 17β-oestradiol and DHA than the group receiving 17β-oestradiol alone ($p=0.05$ and $p=0.02$ respectively) however the difference failed to reach the level of significance required for the contrast following Bonferroni correction ($p<0.01$). Periosteal circumference was significantly greater in the group receiving 17β-oestradiol and DHA than in the group receiving 17β-oestradiol alone ($p=0.01$) (**Table 7**).

Biomechanics

Femurs from ovariectomised animals were significantly less elastic but not significantly weaker upon loading in the anterior-posterior direction than femurs of sham-operated animals ($p=0.01$) (**Table 8**). Treatment with 17β-oestradiol had no statistically significant effect on elastic modulus but was associated with increased femur strength in terms of the amount of energy able to be absorbed prior to breaking ($p=0.01$) and the maximum load able to be withstood prior to breaking ($p=0.005$). The increased femur strength observed in 17β-oestradiol –treated animals was also apparent in animals treated with 17β-oestradiol and DHA.

Osteocalcin

Ovariectomy had no statistically significant effect on plasma concentrations of total, carboxylated or undercarboxylated osteocalcin (**Table 9**). However, dietary DHA supplementation was associated with a higher plasma concentration of carboxylated osteocalcin and a lower plasma concentration of undercarboxylated osteocalcin compared to untreated ovariectomy ($p=0.02$). Treatment with 17β-oestradiol was

associated with lower total plasma osteocalcin concentration compared to untreated ovariectomy (p=0.01).

Interleukin-6

There was a significant interaction between treatment with oestrogenic compounds and DHA supplementation on plasma IL-6 concentrations (p=0.03). Treatment with 17β-oestradiol in conjunction with DHA supplementation resulted in significantly lower plasma IL-6 concentrations compared to 17β-oestradiol treatment without DHA supplementation (p=0.01) (**Table 9**, **Figure 1**).

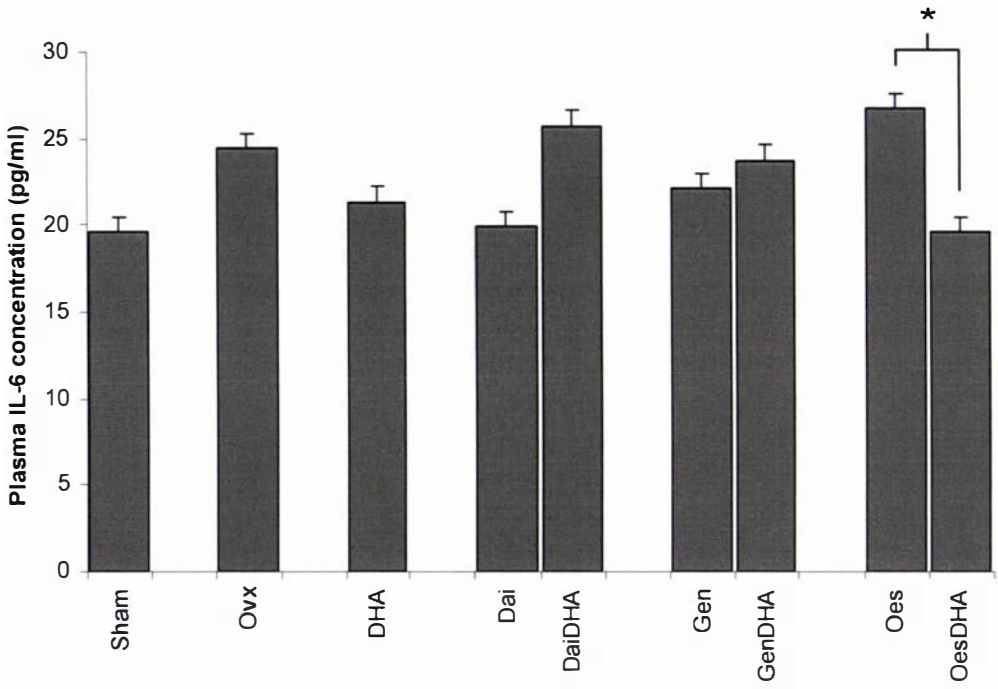

Figure 1 Plasma concentration of IL-6 (pg/ml) in ovariectomised female rats. Rats were treated with DHA (0.5g/kg body weight/day) or oestrogenic compound (genistein or daidzein 20mg/kg body weight/day, 17β-estradiol 1μg/day) or a combination of oestrogenic compounds and DHA for 18-weeks. Plasma IL-6 concentration was measured by ELISA. Results are expressed as the mean ± SE for each study group. Difference between groups marked with * was statistically significant at p<0.01.
Note: The difference in plasma IL6 concentration between animals treated with daidzein without DHA compared to daidzein with DHA failed to reach statistical significance (p=0.07).

Table 7 *Trabecular and cortical bone mineral content (BMC), area (BA) and density (BMD and periosteal and endosteal circumferences of right tibia*

	Sham	Ovariectomised									P-values			
	No Oest. No DHA	No Oest. No DHA	Oest. cmpd			No Oest.	Combined treatment				*Effect of ovx*	*Effect of treatment*		
	Sham	OVX	Dai	Gen	Oes	DHA	Dai+ DHA	Gen+ DHA	Oes+ DHA	SE	*Ovx*	*Oest. Cmpd*	*DHA*	*Oest. Cmpd × DHA*
Trab BMC	5.69	3.47	4.11	4.16	5.80*	4.84*	4.58	4.34	6.13	0.13	*<0.0001*	*<0.0001*	*0.008*	*0.45*
Trab BA	9.5	10.2	9.8	9.5	10.2	10.1	10.3	9.4	10.5	0.15	*0.09*	*0.44*	*0.17*	*0.63*
Trab BMD	593	338	421	437	570*	482	453	459	586	11.4	*<0.0001*	*<0.001*	*0.02*	*0.29*
Cort BMC	6.9	6.8	6.7	6.9	7.0	6.9	6.7	6.8	7.2	0.06	*0.82*	*0.37*	*0.14*	*0.23*
Cort BA	5.07	5.12	5.00	5.10	5.08	5.15	5.00	5.07	5.28	0.04	*0.85*	*0.42*	*0.20*	*0.27*
Cort BMD	1322	1331	1314	1322	1341	1326	1320	1315	1337	3.6	*0.38*	*0.09*	*0.99*	*0.61*
Periosteal circ.	10.05	10.13	10.05	10.11	10.01	10.16	10.08	10.05	10.21[B]	0.04	*0.95*	*0.49*	*0.06*	*0.15*
Endosteal circ.	5.99	6.24	6.14	6.12	5.96*	6.13	6.12	6.05	6.08	0.04	*0.11*	*0.13*	*0.61*	*0.42*

Female rats were ovariectomised (ovx) or sham- operated and treated with daidzein (DAI, 20mg/kg body weight/day), genistein (GEN, 20mg/kg body weight/day), 17β-oestradiol (OES, 1μg/day) or docosahexaenoic acid (DHA, 0.5g/kg body weight/day) or a combination of oestrogenic compound and DHA for 18 weeks. Tibiae were dissected from euthanased animals following the 18-week treatment period. Measurements were made by pQCT. Trabecular BMC, BA and BMD were measured 5mm from the proximal end of the tibia. Cortical BMC, BA and BMD and periosteal and endosteal circumferences were determined at a slice taken 50% along the length of the tibia. Results are expressed as the mean for each study group.

Main Effects Contrasts*: The effect of each of the three oestrogenic compounds was contrasted with the effect of untreated ovariectomy (OVX). The effect of DHA treatment was contrasted with the effect of untreated ovariectomy.*

* *Significantly different from OVX at p<0.01 (Contrast made for main effects only)*

Combined Treatment Contrasts: *Contrasts were constructed for each combined treatment of oestrogenic compound +DHA compared to treatment with the oestrogenic compound alone and DHA alone.*

[A] *Significantly different from DHA alone at p<0.01 (Contrast made for combined treatment only)*

[B] *Significantly different from oestrogenic compound alone at p<0.01 (Contrast made for combined treatment only)*

Table 8 *Biomechanical properties of right femurs*

	Sham	Ovariectomised									*P-values*			
	No Oest. No DHA	No Oest. No DHA	Oest. Cmpd			No Oest.	Combined treatment				*Effect of ovx*	*Effect of treatment*		
	Sham	OVX	Dai	Gen	Oes	DHA	Dai+ DHA	Gen+ DHA	Oes+ DHA	SE	*Ovx*	*Oest. Cmpd*	*DHA*	*Oest. Cmpd × DHA*
Max load (N)	199.5	196.9	188.1	198.2	212.3*	192.5	183.6	195.6	212.2Â	2.72	*0.19*	*0.008*	*0.06*	*0.30*
Elastic modulus (N/mm2)	391	693	490	506	549	465	414	486	457	131	*0.01*	*0.37*	*0.04*	*0.93*
Energy (J)	0.18	0.17	0.16	0.17	0.18*	0.16	0.16	0.16	0.18Â	0.003	*0.44*	*0.03*	*0.02*	*0.56*

Female rats were ovariectomised (ovx) or sham- operated and treated with daidzein (DAI, 20mg/kg body weight/day), genistein (GEN, 20mg/kg body weight/day), 17β-oestradiol (OES, 1μg/day) or docosahexaenoic acid (DHA, 0.5g/kg body weight/day) or a combination of oestrogenic compound and DHA for 18 weeks. Femurs were dissected from euthanased animals following the 18-week treatment period and biomechanical properties of the femurs were determined by three-point bending. Results are expressed as the mean for each study group.

Main Effects Contrasts*: The effect of each of the three oestrogenic compounds was contrasted with the effect of untreated ovariectomy (OVX). The effect of DHA treatment was contrasted with the effect of untreated ovariectomy.*

* *Significantly different from OVX at p<0.01(Contrast made for main effects only)*

Combined Treatment Contrasts: *Contrasts were constructed for each combined treatment of oestrogenic compound +DHA compared to treatment with the oestrogenic compound alone and DHA alone.*

A *Significantly different from DHA alone at p<0.01(Contrast made for combined treatment only)*

B *Significantly different from oestrogenic compound alone at p<0.01(Contrast made for combined treatment only)*

Table 9 *Final plasma concentrations of IL-6 and osteocalcin*

	Sham	Ovariectomised									P-values			
	No Oest. No DHA	No Oest. No DHA	Oest. Cmpd			No Oest.	Combined treatment				*Effect of ovx*	*Effect of treatment*		
	Sham	OVX	Dai	Gen	Oes	DHA	Dai+ DHA	Gen+ DHA	Oes+ DHA	SE	*Ovx*	*Oest. Cmpd*	*DHA*	*Oest. Cmpd × DHA*
IL-6 (pg/ml)	19.6	24.4	19.9	22.1	26.7	21.3	25.7	23.7	19.6[B]	0.9	*0.13*	*0.99*	*0.45*	*0.03*
Carboxylated osteocalcin (ng/ml)	1762	1765	1767	1766	1754	1773*	1787	1780	1762	16.8	*0.19*	*0.16*	*0.02*	*0.88*
Undercarboxylated osteocalcin (ng/ml)	478	475	473	474	485	467*	453	460	478	16.7	*0.19*	*0.16*	*0.02*	*0.88*
Total osteocalcin (ng/ml)	2313	2285	2309	2311	2246*	2310	2279	2293	2283	44.8	*0.20*	*0.08*	*0.38*	*0.21*
% undercarboxylated of total osteocalcin (%)	21.4	21.8	21.2	21.2	21.8	20.9*	20.3	20.6	21.4	0.35	*0.21*	*0.14*	*0.02*	*0.89*

Female rats were ovariectomised (ovx) or sham- operated and treated with daidzein (DAI, 20mg/kg body weight/day), genistein (GEN, 20mg/kg body weight/day), 17β-oestradiol (OES, 1μg/day) or docosahexaenoic acid (DHA, 0.5g/kg body weight/day) or a combination of oestrogenic compound and DHA for 18 weeks. Plasma concentrations of IL-6 and osteocalcin were determined by ELISA in blood samples taken at the end of the 18-week treatment period. Results are expressed as the mean for each study group.

Main Effects Contrasts*: The effect of each of the three oestrogenic compounds was contrasted with the effect of untreated ovariectomy (OVX). The effect of DHA treatment was contrasted with the effect of untreated ovariectomy.*

* *Significantly different from OVX at p<0.01 (Contrast made for main effects only)*

Combined Treatment Contrasts: *Contrasts were constructed for each combined treatment of oestrogenic compound +DHA compared to treatment with the oestrogenic compound alone and DHA alone.*

[A] *Significantly different from DHA alone at p<0.01(Contrast made for combined treatment only)*

[B] *Significantly different from oestrogenic compound alone at p<0.01 (Contrast made for combined treatment only)*

Discussion

Ovariectomy resulted in decreased BMC and BMD in both the lumbar spine and femur. The effects of ovariectomy were particularly apparent in trabecular bone. Treatment with 17β-oestradiol and to a lesser extent, DHA, provided some protection against the ovariectomy-induced decline in BMC.

Structurally, femurs of 17β-oestradiol-treated animals were stronger and had greater BMC than those of untreated ovariectomised controls. Treatment with 17β-oestradiol was associated with lower total plasma osteocalcin concentration compared to ovariectomised controls. Osteocalcin is a bone matrix protein produced by osteoblasts, odontoblasts and hypertrophic chondrocytes [8]. It is released into the bloodstream during new bone formation [9] and fragments of osteocalcin are released during osteoclastic bone resorption [10]. Total circulating osteocalcin concentration serves as a marker of bone remodelling rate with a lower concentration indicative of a lower rate of bone turnover [10]. The lower circulating concentration of total osteocalcin observed with 17β-oestradiol treatment both in the present study in ovariectomised rats and previously in post-menopausal women [11] suggests 17β-oestradiol treatment may reduce bone turnover.

In women, circulating Il-6 concentration increases post-menopause and decreases following oestrogen replacement therapy [12]. In the present study, plasma IL-6 concentration was not significantly different in ovariectomised rats compared to sham, or in 17β-oestradiol –treated animals compared to untreated ovariectomy. In rats, responsiveness of Il-6 to oestrogen is tissue specific. For instance, in vascular smooth muscle cells from rats, Il-6 gene expression is constitutive and not induced by oestrogen [13]. Similarly, treatment of rats with the oestrogenic compound, diethylstilbesterol resulted in an increase in Il-6 mRNA levels in the uterus but not in bone [14]. This may indicate that large changes in Il-6 expression need to occur before the change is reflected in circulating Il-6 concentration.

Both 17β-oestradiol and DHA treatments were associated with significantly higher trabecular BMC and femur BMC and BMD at week 18 compared to untreated ovariectomy. Unlike, 17β-oestradiol however, femurs from DHA-treated animals

were not significantly stronger than those from untreated ovariectomised animals. As neither endosteal circumference nor total plasma osteocalcin concentration were significantly different in DHA-treated animals compared to ovariectomised controls, DHA may have maintained bone mass by a different mechanism than that of 17β-oestradiol. Although total plasma osteocalcin was unchanged with DHA treatment, mean plasma concentration of carboxylated osteocalcin was greater, and mean plasma concentration of undercarboxylated osteocalcin was lower, in DHA-treated compared to untreated ovariectomised controls. Gamma-carboxylation of osteocalcin is essential for hydroxyapatite binding to bone matrix and hence mineralisation of bone. Gamma-carboxylase requires both vitamin K and phospholipids for its activity [15]. A higher plasma concentration of carboxylated osteocalcin suggests that DHA treatment may have promoted new bone formation.

Combined treatment with 17β-oestradiol and DHA resulted in greater F BMC at week 18 compared to either treatment alone. There was a statistically significant interaction between oestrogenic compound and DHA treatments for F BMC. This may indicate DHA and 17β-oestradiol acted synergistically to promote greater BMC. Alternatively, it may reflect a divergent effect of co-treatment with DHA and phytoestrogens as opposed to 17β-oestradiol as combined treatment with DHA and either daidzein or genistein resulted in slightly, but not significantly, lower BMC than DHA treatment alone. There was also evidence of possible interactions between oestrogenic compounds and DHA on both plasma IL-6 concentration and the percentage of n-3 LCPUFAs in RBCs which again may be a result of divergent effects of phytoestrogens compared to 17β-oestradiol when co-administered with DHA. As co-treatment with 17β-oestradiol and DHA was associated with a lower plasma IL-6 concentration and a higher percentage of n-3 LCPUFA in RBC, combined treatment with 17β-oestradiol and DHA may have a beneficial effect in reducing inflammatory status. This may have implications for the prevention of other inflammatory diseases.

The effect of phytoestrogens on bone post-ovariectomy is controversial. Although many studies have been published in this field, considerable heterogeneity in terms of the composition and dose of phytoestrogen supplements that have been administered and the type of animal model used have likely contributed to the

considerable inconsistency in results obtained. In the present study, genistein supplementation had a weak protective effect against ovariectomy-induced loss of LS BMC in the first 5 weeks following ovariectomy. However, the effect was transient and no beneficial effect of genistein on bone mass was evident at week 18 following ovariectomy. Three short-term studies have reported bone mass-preserving effects of genistein and equol (the main metabolite of daidzein) in the first 2-3 weeks following ovariectomy [16-18]. Beneficial effects on bone mass of daidzein and/or genistein administered in similar doses to those in the present study have been reported up to 22 weeks post-ovariectomy in animals fed a low calcium diet [19-21]. It is possible that when a calcium-adequate diet is fed, such as in the present study, the effects of genistein and daidzein on bone mass are minimal in the longer term. Daidzein supplementation was associated with reduced body weight gain post-ovariectomy. A similar result has previously been shown following soy isoflavone consumption and is believed to be a result of a reduction in food utilisation rate [22].

In the present study combined supplementation with genistein or daidzein and DHA had no beneficial effect on BMC above that of DHA treatment alone. Watkins *et. al.* (2005) observed a reduction in serum concentrations of pyridinoline, a marker of bone resorption, in growing ovariectomised rats fed a low calcium diet (0.11% of diet) supplemented with menhaden oil (a source of n-3 LCPUFAs) and a soy isoflavone-containing protein supplement. Although treatment with n-3 LCPUFAs alone had a beneficial effect on bone mass in the Watkins *et. al.* (2005) study, combined soy isoflavone/n-3 LCPUFA supplementation failed to further augment bone mass [7].

In conclusion, although combined treatment with DHA and 17β-oestradiol was more efficacious than either treatment alone in ameliorating the effects of ovariectomy on BMC, there was no evidence of a beneficial effect of combined DHA and daidzein or genistein treatment on BMC post-ovariectomy. Further work is required in order to determine the mechanism of action of DHA on bone and to clarify the interaction between the bioactivities of 17β-oestradiol and DHA.

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CHAPTER 6

Ovariectomy and omega-3 fatty acid supplementation alter the profiles of inflammatory and pro-resolving lipid mediators in murine bone marrow

Both EPA and DHA are further metabolised to a range of lipid mediators which have demonstrated bioactivity in a variety of tissues. Whether these lipid mediators have a role in the normal regulation of bone remodelling is unknown. The objective of the study presented in this chapter was to determine whether these lipid mediators are present in bone marrow.

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Abstract

Newly-described lipoxygenase (LOX)-generated lipid mediators namely resolvins, lipoxins, protectins and other docosanoids have anti-inflammatory and pro-resolving activities. We aimed to determine if these LOX-generated lipid mediators are present in rat bone marrow and whether the profile of LOX lipid mediators alters following ovariectomy or dietary supplementation with eicosapentaenoic ethyl ester (EPA, 20:5n-3) or docosahexaenoic ethyl ester (DHA, 22:6n-3). Six-month old Sprague Dawley rats were either ovariectomised or sham-operated. Shams and one ovariectomised group were fed a diet devoid of n-3 LCPUFAs. The remaining two ovariectomised groups were fed a diet supplemented with 0.5g EPA or DHA ethyl ester/kg body weight/day for four months. Following euthanasia, bone marrow from tibia and femurs was collected and analysed using LC/MS/MS for the presence of LOX-pathway lipid mediators derived from arachidonic acid (AA, 20:4n-6), EPA and DHA. LOX-derived lipid mediators including lipoxins, resolvin E1 and protectin D1 were identified in bone marrow by the presence of diagnostic ions in their corresponding MS-MS spectra. A higher proportion of AA-derived relative to DHA-derived mediators were present in bone marrow of ovariectomised compared to sham-operated rats. Dietary DHA or EPA ethyl ester supplementation increased the proportion of LOX mediators in bone marrow generated from DHA or EPA respectively. This is the first report documenting the presence of pro-resolving lipid mediators in murine bone marrow. Given the known bioactivities of LOX-derived lipid mediators, the change in the profile of LOX family products post-ovariectomy and following EPA and DHA ethyl ester supplementation may be of physiological relevance.

Introduction

Lipid mediators, such as prostaglandins and other eicosanoids derived from n-6 long chain polyunsaturated fatty acids (LCPUFAs) by the action of cyclooxygenases (COX) and lipoxygenases (LOX), have a major role in initiating the inflammatory process [1-3]. The recent discovery of several new families of LOX-generated eicosanoid and docosanoid lipid mediators derived from both n-6 and n-3 LCPUFAs [4, 5] which have potent anti-inflammatory and pro-resolving activities [6, 7], has

considerably advanced our understanding of how the inflammatory process is naturally resolved.

Eicosapentaenoic acid (EPA, 20:5n-3), docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) are converted to lipid hydroperoxides by LOX-initiated reactions. These hydroperoxides are further transformed, via epoxide intermediates, to potent bioactive lipid mediators known as resolvins, lipoxins, and protectins. LOX-derived lipid mediators inhibit the inflammatory process and promote the resolution of inflammation and the return to normal homeostasis [8].

Chronic inflammation is a major cause of morbidity and mortality in Western countries. Susceptibility to chronic inflammatory disease is influenced by lifestyle and life-stage. In women, the risk of developing many chronic inflammatory diseases such as Alzheimer's disease, osteoporosis and possibly heart disease increases post-menopause [9-12]. LCPUFA metabolism is disrupted following menopause. This results in decreased tissue and blood concentrations of very long chain PUFAs [13, 14], the precursors for resolvin, protectin and lipoxin biosynthesis. As resolvins and docosanoids are potent vasodilators [15], protectin D1 promotes neural cell survival [16] and both resolvins and lipoxins strongly inhibit inflammation-induced bone resorption [17, 18], this may have consequences for the pathogenesis of heart disease, Alzheimer's Disease and osteoporosis post-menopause.

Ovariectomy in rodents and menopause in women not only results in reduced oestrogen levels but also leads to changes in the glucocorticoid response to stress [19-21]. Oestrogens and glucocorticoids regulate the biosynthesis of COX-derived and LOX-derived eicosanoids [22-27]. Eicosanoids regulate the proliferation of stem cells and progenitors as well as the differentiation of these progenitors into neural, immune and bone cells [28-33]. Changes in progenitor cell number and fate occur following surgical menopause [34-36] and altered eicosanoid synthesis is likely to contribute to these changes. As the docosanoid protectin D1 promotes neural cell survival [16] and lipoxins regulate neural stem cell proliferation and differentiation [33], it is of interest to determine whether the profile of these newly-described LOX-generated lipid mediators also changes post-ovariectomy.

Bone marrow is a lipid-rich medium containing stem cells and progenitors for a diverse range of cell types including bone cells and cells of the immune system; all of which respond to pro-inflammatory signalling. A change in the regulatory environment within bone marrow has the potential to influence a wide range of tissues and systems within the body. The objectives of the present study were to establish whether resolvin pathway lipid mediators are present in rat bone marrow, to determine whether the profile of LOX products in rat bone marrow alters following ovariectomy and to ascertain whether dietary intake of the precursor LCPUFAs impacts the profile of LOX-derived lipid mediators in rat bone marrow. Here we report that a higher proportion of AA-derived lipid mediators relative to DHA derived lipid mediators were present in bone marrow in ovariectomised animals compared to sham-operated controls. Increased dietary intake of ethyl esters of DHA or EPA were associated with an increased proportion of LOX-derived lipid mediators generated from DHA or EPA respectively in bone marrow.

Method

Animals

Female Sprague-Dawley rats aged 7 months, were randomly assigned to one of four groups and either ovariectomised (OVX) (1 group, n=10; 2 groups, n=12) or sham operated (1 group, n=10) under general anaesthetic (isofluorane). Sham operated animals were anaesthetised and an incision made in the peritoneal wall but the ovaries left intact.

All animals were maintained in individual shoebox cages at 22°C (± 2°C) with a 12h/12h light/dark cycle in a dedicated room. The study was approved by Massey University Animal Ethics Committee.

Diets

Animals were acclimated to a nutritionally balanced, semi-synthetic diet comprising 14% caseinate, 5% cellulose, 4% corn oil, 0.5% calcium, 60% starch with added vitamins and minerals for four weeks prior to ovariectomy (week -4 to week 0). The diet formulation was based on AIN93M [37] with added vitamins and minerals as necessary to compensate for the nutrient content of local ingredients [38]. The type

of oil in the diet was also altered from soybean (as stipulated for AIN93M) to corn oil as soybean oil is a source of n-3 LCPUFAs. The sham-operated ("SHAM", n=10) and ovariectomised control ("OVX", n=10) groups were maintained on this diet for the 4-month study period. Immediately following ovariectomy, the experimental groups (n=12 per group) were fed diets in which some of the corn oil was replaced with ethyl esters of either EPA (90% purity, 01177A-E90 Sanmark LLC, USA), or DHA (80% purity, 01177B-E80 Sanmark LLC, USA) at a dose of 0.5 g/kg body weight/day. All diets contained 4% total fat and at least 2% corn oil, an amount in excess of the minimum required to prevent n-6 LCPUFA deficiency (1%). Diets were randomly sampled and analysed in order to confirm nutrient content. Diet compositions are outlined in **Table 1**.

Table 1 *Ingredient composition (% air-dry weight) of control and experimental diets*

	Percentage of Diet			
	SHAM	**OVX**	**EPA**	**DHA**
Cornstarch	59.7	59.7	59.7	59.7
Sodium Caseinate	14	14	14	14
Sucrose	6	6	6	6
Cellulose	5	5	5	5
Vitamins[a]	5	5	5	5
Minerals (excl Ca)[b]	5	5	5	5
Calcium Carbonate	1.3	1.3	1.3	1.3
Corn Oil [c]	4	4	3.3 – 3.1	3.3 – 3.1
Eicosapentaenoic acid ethyl ester [c]	0	0	0.7 – 0.9	0
Docosahexaenoic acid ethyl ester [c]	0	0	0	0.7 – 0.9

[a] Supplying (mg/kg diet) retinol acetate 5.0, $_{DL}$-α-tocopherol acetate 200.0, menadione 3.0, thiamine hydrochloride 5.0, riboflavin 7.0, pyridoxine hydrochloride 8.0, D-pantothenic acid 20.0, folic acid 2.0, nicotinic acid 20.0, D-biotin 1.0, myo-inositol 200.0, choline chloride 1500; (μg/kg diet) ergocalciferol 25.0, cyanocobalamin 50.0.

[b] Supplying (g/kg diet) chloride 7.79, magnesium 1.06, phosphate 4.86, potassium 5.24, sodium 1.97; (mg/kg diet) chromium 1.97, copper 10.7, iron 424, manganese 78.0, zinc 48.2; (μg/kg diet) cobalt 29.0, iodine 105.0, molybdenum 152.0, Selenium 151.0

[c] LCPUFA ethyl ester dose was 0.5g/kg rat body weight/day. Percentage of LCPUFA ethyl ester in diets increased and percentage of corn oil decreased as the body weight of animals increased over the trial period. Values given are the minimum and maximum amounts of LCPUFA ethyl ester provided at trial commencement and trial completion.

LCPUFAs and corn oil were blended into the experimental diet on a daily basis in order to prevent PUFA oxidation. Body weights of all animals were measured weekly, and the amount of LCPUFA and corn oil added to each test diet was adjusted according to the mean body weight of animals in each treatment group. The SHAM group was fed *ad libitum*. The food intake of ovariectomised animals was limited to that of SHAM in order to reduce ovariectomy-induced weight gain. All

animals had *ad libitum* access to deionised water throughout the study period. Daily food intake was recorded.

Sample collection

Marrow from the tibia and femur was extracted by removing the distal and proximal epiphyses to expose the marrow-filled diaphysis, centrifuging at 3000 rpm for 5 mins at 4˚C and removing the marrow-free bone with forceps. Marrow was immediately frozen and stored at -80°C.

Extraction of LOX products from bone marrow

Methanol (1 ml) was added to the bone marrow immediately upon removal from frozen storage. Marrow was thawed and ground with a tissue grinder and a further 2 ml of methanol added. Samples were mixed by vortex and proteins were precipitated by allowing tostand at -20˚C overnight. Cellular material was removed by centrifuging at 2000 rpm for 20 mins at 2˚C. Milli-Q water (30 ml) was added and samples were acidified to pH 3.5 with 1N HCl.

C18 SPE columns (Alltech, Deerfield, IL Cat # 405250 Extract-Clean# Ev SPE 500 mg) were primed with methanol then washed with 20ml methanol, followed by 20 ml of Milli-Q water. Samples were loaded and washed with 20 ml water, 8 ml of hexane eluted with 8 ml of methyl formate. The methyl formate fraction was collected and dried under vacuum to remove water. Samples were resuspended in methanol and stored -80°C prior to analysis.

Lipid Mediator Lipidomics: Identification and analysis of LOX products

Liquid chromatography-tandem mass spectrometer (LC/MS/MS) identification was acquired with a LCQ (ThermoFinnigan, San Jose, CA) quadrupole ion trap spectrometer system equipped with an electrospray ionization probe. Samples were suspended in mobile phase and injected into the HPLC component, which consisted of a SpectraSYSTEMS P4000 (ThermoFinnigan, San Jose CA) quaternary gradient pump, with a Thermo Electron BDS Hypersil C18 (100 x 2 mm, 5 μm) column

(ThermoFisher Scientific, Waltham, MA). The column was eluted at a flow rate of 0.2 ml/min with methanol/water/acetic acid (65:34.99:0.01, v/v/v) from 0 to 8 min, and then a gradient increasing to 100 percent methanol from 8.01 to 30 min.

Samples were scanned for the presence of 5-hydroxyeicosatetraenoic acid (5-HETE), 12-HETE, 15-HETE, leukotriene B4 (LTB4), lipoxins A4 and B4 (LXA4 and LXB4), 14-hydroxdocosahexaenoic acid (14-HDHA), 17-HDHA, resolvin D1 (RvD1), protectin D1 (PD1), 5-hydroxyeicosapentaenoic acid (5-HEPE), 15-HEPE, 18-HEPE, RvE1 and RvE2. Criteria for identification were: LC retention time and UV chromaphore matched with reference standard, and a minimum of 4 to 6 fragment diagnostic ions on the MS/MS spectrum matched that of reference standard.

Statistical Analysis

Comparisons between groups were made by one-way ANOVA with post-hoc Tukey testing using Minitab® 14, Minitab Inc., Pennsylvania, USA. A p-value of ≤ 0.05 was considered significant.

Results

Dietary intake

In the LCPUFA-supplemented animals, daily intake of EPA or DHA ethyl ester was 160 – 180 mg per animal per day. There were no significant differences among groups in terms of the amount of food consumed per day with all animals consuming 18-20 g per day.

Identification of LOX products in bone marrow

Lipoxygenase products from AA, EPA and DHA were identified in bone marrow samples from all groups. In the SHAM, OVX and DHA groups the AA derived products LTB4, LXA4 and LXB4, and the monohydroxy 5-, 12- and 15-HETE were identified. LC/MS/MS spectra are provided in **Figure 1**. Although all three HETEs and LTB4 were also identified in the EPA supplemented group, neither LXA4 nor LXB4 were clearly identified in any sample from this group **(Figure 2)**.

Figure 1

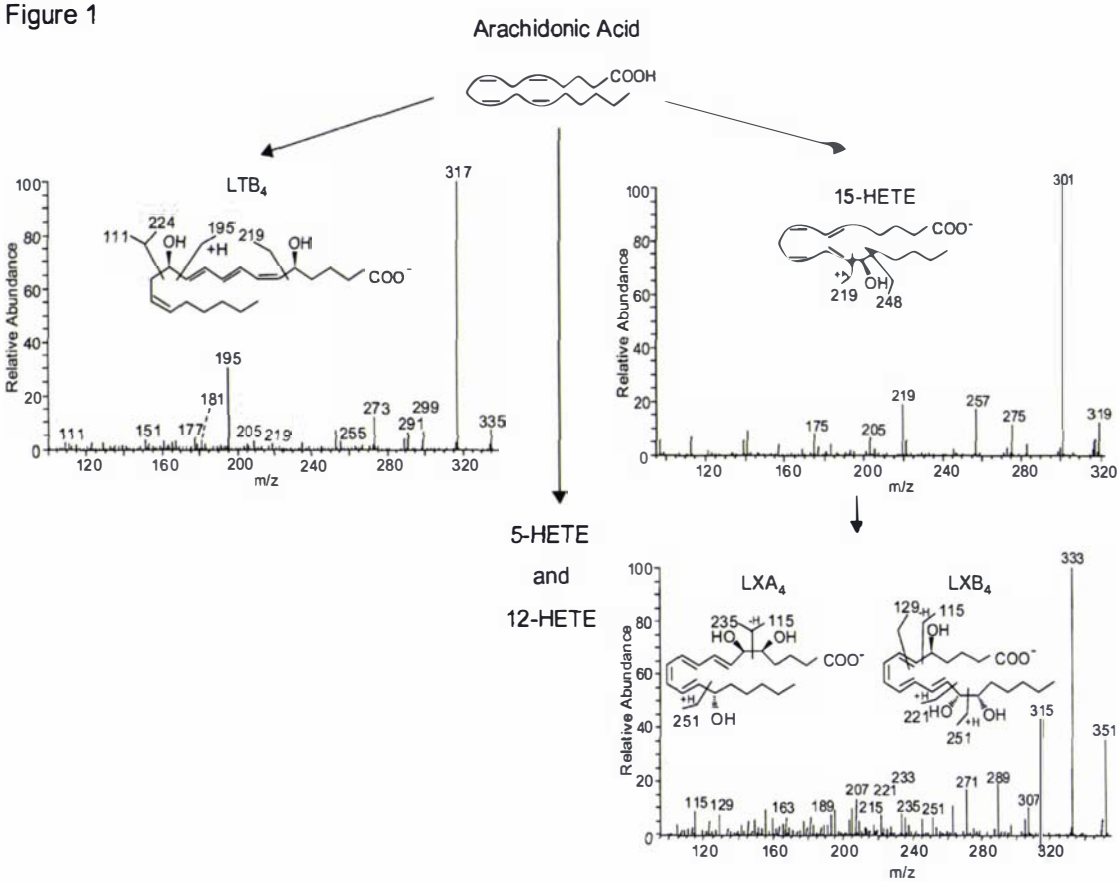

Figure 1 Lipoxygenase-catalysed lipid mediator formation from arachidonic acid and LC-MS/MS spectra for lipoxin pathway products.

15-HETE MS/MS fragmentation—m/z 319: [M-H]; m/z 301: [M-H] - H_2O; m/z 275: [M-H] - CO_2; m/z 257: [M-H] - H_2O, - CO_2; m/z 248; m/z 219; m/z 205: m/z 248 - CO_2; m/z 175: m/z 219 - CO_2.

LXA4 MS/MS fragmentation—m/z 351: [M-H]; m/z 333: [M-H] - H_2O; m/z 315: [M-H] - $2H_2O$; m/z 307: [M-H] - CO_2; m/z 289: [M-H] - H_2O, - CO_2; m/z 271: [M-H] - $2H_2O$, - CO_2; m/z 251; m/z 235; m/z 233: m/z 251 - H_2O; m/z 215: m/z 251 - $2H_2O$; m/z 207: m/z 251 - CO_2; m/z 189: m/z 251 - H_2O, - CO_2; m/z 115.

LXB4 MS/MS fragmentation—m/z 351: [M-H]; m/z 333: [M-H] - H_2O; m/z 315: [M-H] - $2H_2O$; m/z 307: [M-H] - CO_2; m/z 289: [M-H] - H_2O, - CO_2; m/z 271: [M-H] - $2H_2O$, - CO_2; m/z 251; m/z 233: m/z 251 - H_2O; m/z 221; m/z 215: m/z 251 - $2H_2O$; m/z 207: m/z 251 - CO_2; m/z 189: m/z 251 - H_2O, - CO_2; m/z 163; m/z 129; m/z 115.

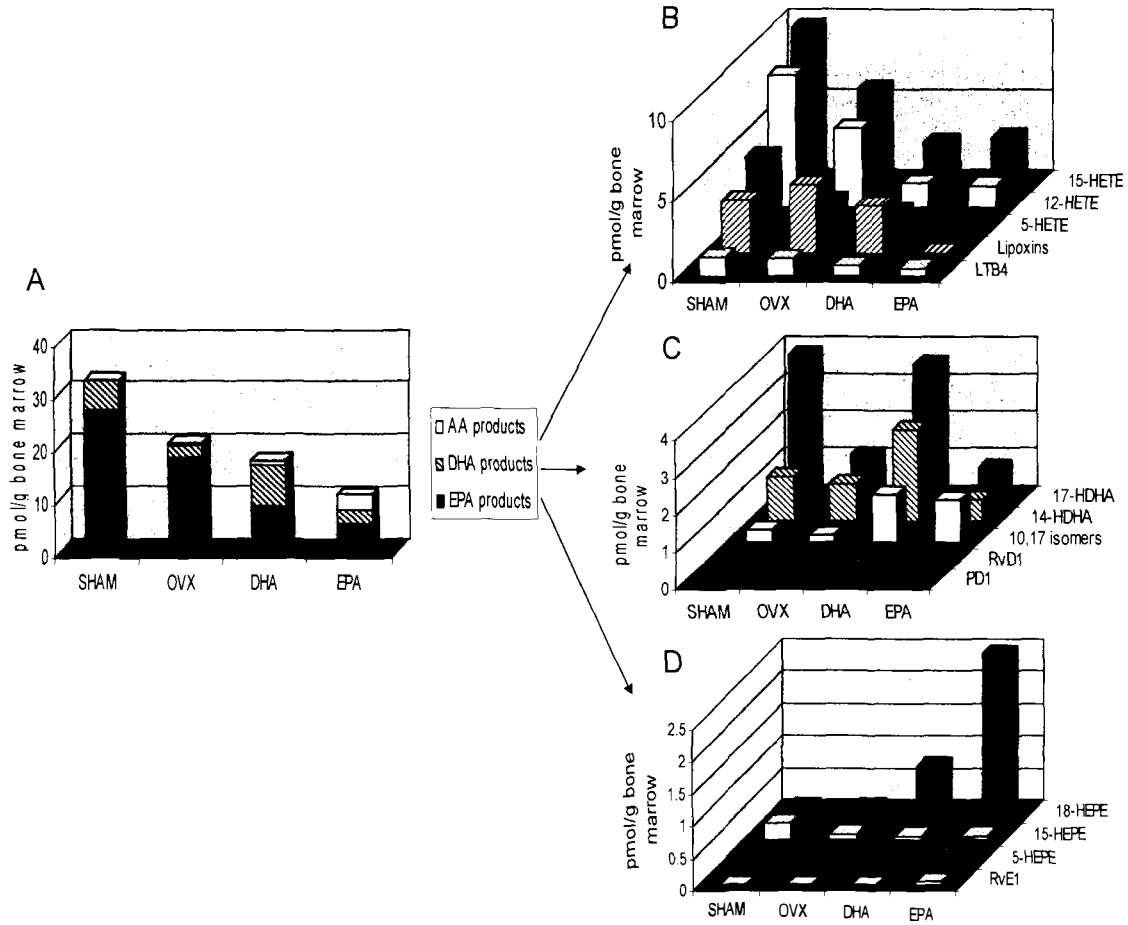

Figure 2 Levels of LOX-generated lipid mediators derived from arachidonic, docosahexaenoic and eicosapentaenoic acids in bone marrow from female rats. ***A*** Total concentrations of lipoxygenase-generated lipid mediators derived from arachidonic, docosahexaenoic and eicosapentaenoic acids and concentrations of specific products of ***B*** arachidonic acid, ***C*** docosahexaenoic acid and ***D*** eicosapentaenoic acid in bone marrow from female rats following sham-operation or ovariectomy and 4 months of consumption of either a diet devoid of n-3 LCPUFAs or a diet supplemented with eicosapentaenoic ethyl ester or docosahexaenoic ethyl ester (0.5 g/kg body weight/day). Bone marrow was obtained from femurs and tibiae at trial completion and lipid mediator lipidomic analysis conducted by LC-MS/MS.

The DHA derived lipid mediators 14-HDHA and 17-HDHA were present in samples from all groups. PD1 was identified in 4 out of the 5 samples from the DHA group and 2 out of the 3 samples each from the OVX and SHAM groups. PD1 was not identified in any sample from the EPA group. Several isomers of 10,17-dihydroxy-docosatriene which did not match the retention time of PD1 were present in all samples. RvD1 was present in some, but not all samples from all four treatment groups (2 out of 3 samples OVX, 1 out of 3 samples SHAM, 4 out of 5 samples DHA and 3 out of 6 samples EPA). LC/MS/MS spectra for DHA-derived lipid mediators are provided in **Figure 3.**

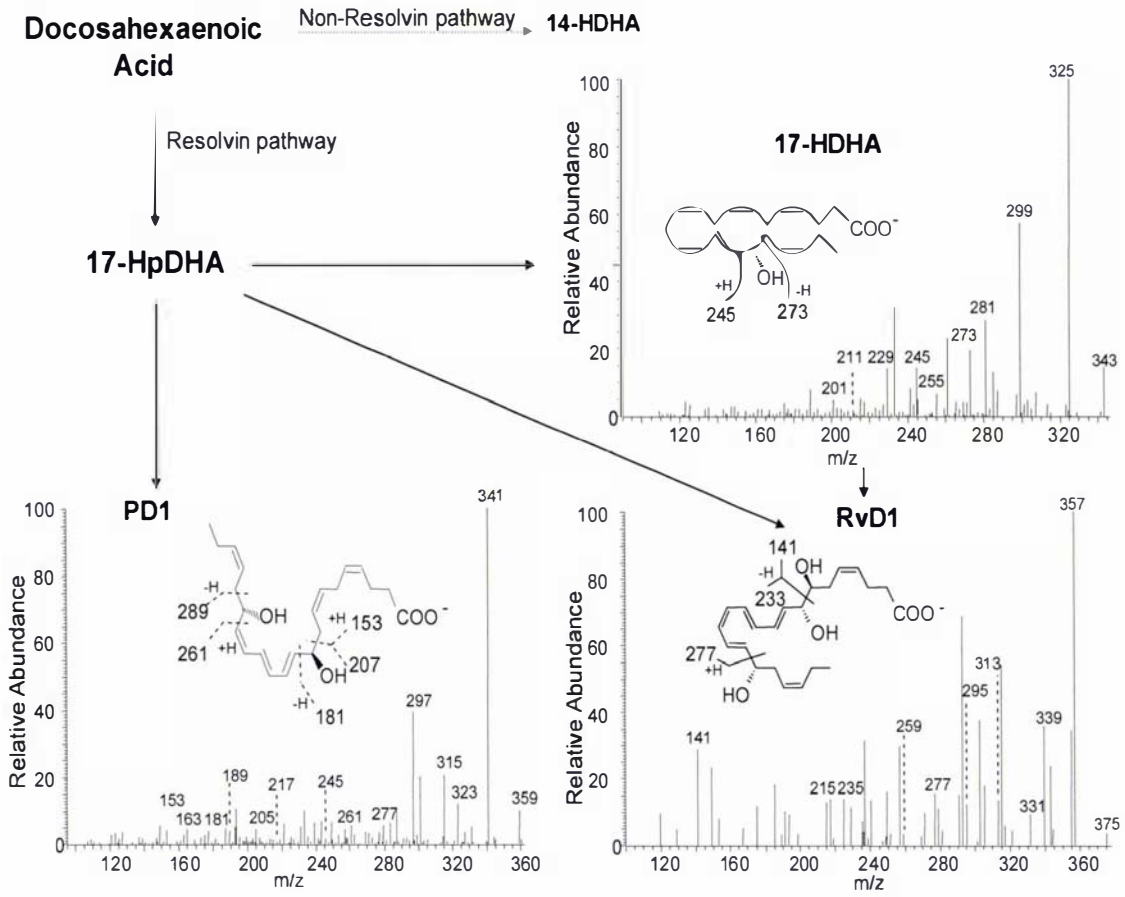

Figure 3 Lipoxygenase-catalysed lipid mediator formation from docosahexaenoic acid and LC-MS/MS spectra for resolvin pathway products.

17-HDHA MS/MS fragmentation—m/z 343: [M-H]; m/z 325: [M-H] - H_2O; m/z 299: [M-H] - CO_2; m/z 281: [M-H] - H_2O, - CO_2; m/z 273; m/z 255: m/z 273 - H_2O; m/z 245; m/z 229: m/z 273 - CO_2; m/z 211: m/z 273 - H_2O, - CO_2; m/z 201: m/z 245 - CO_2.

PD1 MS/MS fragmentation—m/z 359: [M-H]; m/z 341: [M-H] - H_2O; m/z 323: [M-H] - $2H_2O$; m/z 315: [M-H] - CO_2; m/z 297: [M-H] - H_2O, - CO_2; m/z 289; m/z 277: [M-H] - $2H_2O$, - CO_2 - 2H; m/z 261; m/z 245: m/z 289 - CO_2; m/z 217: m/z 261 - CO_2; m/z 205: m/z 207 - 2H; m/z 189: m/z 207- H_2O; m/z 181; m/z 163: m/z 181 - H_2O; m/z 153.

RvD1 MS/MS fragmentation—m/z 375: [M-H]; m/z 357: [M-H] - H_2O; m/z 339: [M-H] - $2H_2O$; m/z 331: [M-H] - CO_2; m/z 313: [M-H] - H_2O, - CO_2; m/z 295: [M-H] - $2H_2O$, - CO_2; m/z 277; m/z 259: m/z 277 - H_2O; m/z 235: m/z 233 + 2H; m/z 233;
m/z 215: m/z 233 - H_2O; m/z 141.

Of the LOX pathway products derived from EPA, 15-HEPE and 18-HEPE were identified in all samples analysed, however 5-HEPE was only present in samples from the EPA group and in 1 sample from the DHA group. Interestingly in all 6 EPA samples as well as the one DHA sample in which 5-HEPE was identified, neither LXA4 nor LXB4 were identified. RvE1 was present in 3 of the 6 samples from the EPA group and in one sample each from the SHAM and DHA groups but was not identified in any sample from the OVX group. RvE2 was not identified in any of

these samples (**Figure 2**). LC/MS/MS spectra for 18-HEPE and RvE1 are provided in **Figure 4**.

Figure 4

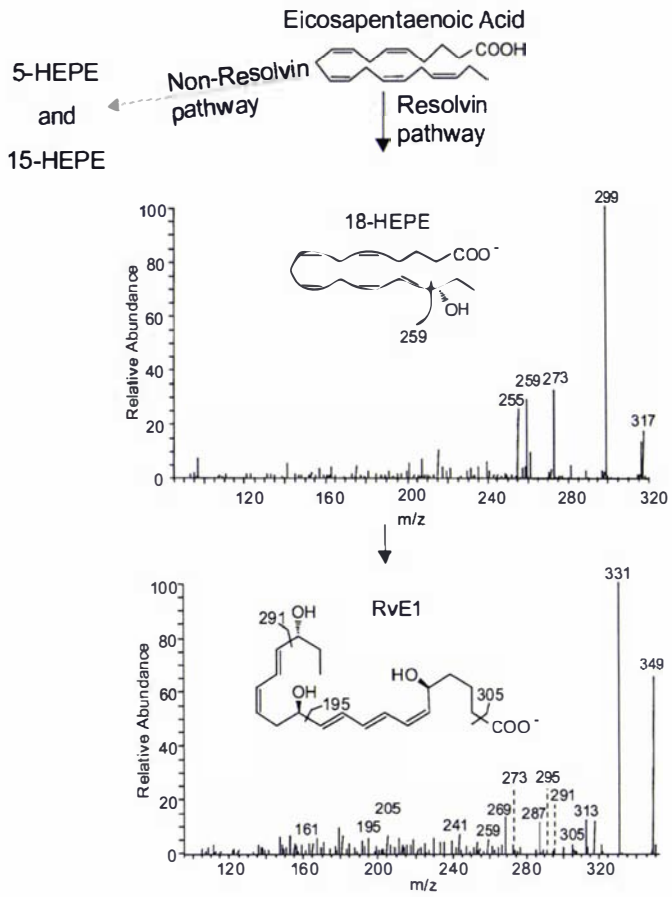

Figure 4 Lipoxygenase-catalysed lipid mediator formation from eicosapentaenoic acid and LC-MS/MS spectra for resolvin pathway products.

18-HEPE MS/MS fragmentation—m/z 317: [M-H]; m/z 299: [M-H] - H_2O; m/z 273; m/z 259; m/z 255 : [M-H] - H_2O, - CO_2.

RvE1 MS/MS Fragmentation—m/z 349: [M-H]; m/z 331: [M-H] - H_2O; m/z 313: [M-H] - $2H_2O$; m/z 305: [M-H] - CO_2; m/z 295 [M-H] - $3H_2O$; m/z 291; m/z 287: [M-H] - H_2O, - CO_2; m/z 273: m/z 291 - H_2O; m/z 269: [M-H] - $2H_2O$, - CO_2; m/z 259: m/z 321 - H_2O, - CO_2; m/z 241: m/z 321 - $2H_2O$, - CO_2; m/z 205: m/z 223 - H_2O; m/z 195; m/z 161: m/z 223 - H_2O, - CO_2.

There was considerable inter-individual variation in terms of the levels of LOX pathway products in bone marrow (**Table 2**) however within each treatment group the relative proportion of EPA, DHA and AA families of bioactive mediators in bone marrow was much more consistent (**Table 3**). For this reason, comparisons were made between groups based on the levels of each product identified as well as on the percentage of total LOX product profiles accounted for by each individual product.

Table 2 *Quantity (pmol/g bone marrow) of LOX mediators in bone marrow*

Metabolite	SHAM	OVX	EPA	DHA	*p-value*
LXA4	3140 [a] (930)	4049 [a] (1621)	ND [b]	2875 [a] (1041)	*0.023*
LXB4	115.6 [a] (18.5)	142.5 [a] (37.7)	ND [b]	75.7 [a] (25.3)	*0.001*
LtB4	1166 [a] (407)	1051 [a] (346)	448 [a] (121)	590 [a] (194)	*0.140*
5-HETE	4583 [a] (1830)	1844 [a,b] (753)	691 [b] (130)	1160 [b] (391)	*0.014*
12-HETE	8249 [a] (4557)	4889 [a] (2656)	1344 [a] (221)	1502 [a] (531)	*0.070*
15-HETE	9817 [a] (4086)	5946 [a,b] (2488)	2935 [b] (277)	2666 [b] (683)	*0.046*
Total AA-derived mediators	27070 [a] (11816)	17922 [a,b] (7148)	5418 [b] (699)	8869 [a,b] (2066)	*0.037*
14-HDHA	1193 [a] (798)	981 [a] (957)	583 [a] (511)	2453 [a] (1918)	*0.701*
17-HDHA	3875 [a] (1602)	1207 [a] (685)	865 [a] (333)	3627 [a] (1097)	*0.066*
RvD1	38.2 [a] (38.2)	89.8 [a] (89.8)	Trace [a]	203 [a] (158)	*0.452*
PD1	31.2 [a] (31.2)	Trace [a]	ND [a]	Trace [a]	*0.200*
Other 10,17 diHDHA (NPD1/PD1 isomers)	326 [a] (211)	186 [a] (80.1)	1115 [a] (163)	1283 [a] (427)	*0.059*
Total Resolvin-pathway DHA-derived mediators	3944 [a] (1615)	1297 [a] (774)	865 [a] (333)	3830 [a] (1201)	*0.072*
Total DHA-derived mediators	5463 [a] (2609)	2464 [a] (1804)	2564 [a] (571)	7566 [a] (2777)	*0.230*
5-HEPE	ND [a]	ND [a]	246.3 [a] (93.2)	19.1 [a] (19.1)	*0.044*
15-HEPE	240 [a] (118)	79.2 [a] (27.0)	53.7 [a] (21.5)	55.1 [a] (40.7)	*0.094*
18-HEPE	135.9 [a] (31.5)	129.2 [a] (77.5)	2580 [b] (247)	778 [a] (339)	*<0.0001*
RvE1	Trace [a]	ND [a]	45.6 [a] (45.6)	Trace [a]	*0.650*
Total Resolvin-pathway EPA-derived mediators	135.9 [a] (31.5)	129.2 [a] (77.5)	2625 [b] (240)	778 [a] (339)	*<0.0001*
Total EPA-derived mediators	376 [a] (147)	208 [a] (104)	2925 [b] (266)	852 [a] (348)	*<0.0001*

Female rats were sham-operated or ovariectomised and fed a diet devoid of n-3 LCPUFAs or a diet supplemented with eicosapentaenoic ethyl ester or docosahexaenoic ethyl ester (0.5 g/kg body weight/day) for 4 months. Bone marrow was obtained from femurs and tibiae at trial completion and lipid mediator lipidomic analysis conducted by LC-MS/MS. Results are expressed as the mean with SE in ( ). Different letters denote significant differences between groups within the same row at $p<0.05$

Table 3 *Percentage of LOX-generated lipid mediators in bone marrow*

	Percent				
	SHAM	**OVX**	**EPA**	**DHA**	***p-value***
% LXA4	10.7 [a,b] (5.04)	20.9 [a] (5.04)	0 [b] (3.57)	21.2 [a] (3.91)	0.006
% LXB4	0.4 [a,b] (0.19)	0.8 [a] (0.19)	0 [b] (0.13)	0.6 [a] (0.14)	0.010
% LtB4	3.8 [a] (1.72)	6.8 [a] (1.72)	4.6 [a] (1.21)	2.9 [a] (1.33)	0.379
% 5-HETE	14.4 [a] (0.78)	9.3 [b] (0.78)	6.2 [c] (0.55)	6.3 [c] (0.60)	<0.0001
% 12-HETE	22.7 [a] (2.04)	22.3 [a] (2.04)	12.07 [b] (1.44)	8.7 [b] (1.58)	<0.0001
% 15-HETE	30.5 [a] (1.80)	29.5 [a] (1.80)	26.9 [a] (1.27)	16.2 [b] (1.39)	<0.0001
% AA-derived mediators	82.7 [a] (5.13)	89.6 [a] (5.13)	49.8 [b] (3.63)	55.9 [b] (3.98)	<0.0001
% 14-HDHA	3.0 [a] (5.78)	2.9 [a] (5.78)	3.8 [a] (4.09)	11.1 [a] (4.48)	0.561
% 17-HDHA	12.1 [a,b] (2.80)	5.3 [a] (2.80)	7.9 [a] (1.98)	19.3 [b] (2.17)	0.005
% 10,17 diHDHA (NPD1/PD1 isomers)	0.8 [a] (1.97)	1.1 [a] (1.97)	10.8 [b] (1.39)	7.8 [a,b] (1.53)	0.002
% DHA-derived mediators	16.1 [a] (5.73)	9.5 [a] (5.73)	22.5 [a] (4.05)	39.0 [b] (4.44)	0.006
% 5-HEPE	0 [a] (0.55)	0 [a] (0.55)	2.0 [b] (0.39)	0.1 [a] (0.43)	0.011
% 15-HEPE	0.7 [a] (0.15)	0.4 [a] (0.15)	0.4 [a] (0.10)	0.2 [a] (0.11)	0.168
% 18-HEPE	0.5 [a] (3.20)	0.5 [a] (3.20)	25.0 [b] (2.26)	4.8 [a] (2.48)	<0.0001
% EPA –derived mediators	1.2 [a] (2.75)	1.0 [a] (2.75)	27.8 [b] (1.94)	5.1 [a] (2.13)	<0.0001
% LtB4 of total LXA4 and LtB4	25.8 [a] (13.61)	26.1 [a] (13.61)	100 [b] (9.62)	27.84 [a] (10.54)	<0.0001
Ratio AA:EPA-derived mediators	69.8 [a] (11.22)	93.7 [a] (11.22)	1.9 [b] (7.94)	25.4 [b] (8.69)	<0.0001
Ratio AA:DHA-derived mediators	5.2 [a] (1.81)	12.6 [b] (1.81)	2.6 [a] (1.28)	1.6 [a] (1.40)	0.002
Ratio DHA:EPA-derived mediators	13.6 [a] (1.46)	9.3 [a,b] (1.46)	0.88 [c] (1.03)	5.9 [b] (1.26)	<0.0001

Female rats were sham-operated or ovariectomised and fed a diet devoid of n-3 LCPUFAs or a diet supplemented with eicosapentaenoic ethyl ester or docosahexaenoic ethyl ester (0.5 g/kg body weight/day) for 4 months. Bone marrow was obtained from femurs and tibiae at trial completion and lipid mediator lipidomic analysis conducted by LC-MS/MS. Results are expressed as the mean with SE in ( ). Different letters denote significant differences between groups within the same row at $p<0.05$.

Impact of Ovariectomy

There were no significant differences between the OVX and SHAM groups in the amounts of any of the AA, DHA or EPA lipoxygenase product profiles or in terms of

total amounts of all AA, DHA or EPA lipoxygenase products **(Table 2)**. The EPA derived products accounted for ~1% of total LOX profiles in both the SHAM and OVX groups **(Table 3)**. The ratio of AA pathway relative to EPA LOX pathway products in the OVX group was not significantly different from SHAM. In contrast, the ratio of AA products relative to DHA products was significantly greater in OVX compared to SHAM (p=0.05) (**Table 3**). The percentage of 5-HETE of total identified LOX pathways was significantly lower in OVX compared to SHAM (p=0.002).

Impact of Dietary LCPUFA Supplementation

Total levels of all EPA LOX products identified as well as the percentage of total LOX products derived from EPA were significantly higher compared to all other groups in the EPA-supplemented group (EPA vs SHAM p= 0.0002, EPA vs OVX p=0.0001, EPA vs DHA p=0.0004) (**Table 2**). The percentage of total LOX pathway products accounted for by 18-HEPE and 5-HEPE, but not 15-HEPE, was significantly higher in the EPA group compared to all other groups (18-HEPE: p=0.0002 for all; 5-HEPE: EPA vs OVX or SHAM p=0.047, EPA vs DHA p=0.024). The percentage of isomers of 10,17 dihydroxy-docosatrienes in bone marrow was significantly higher in EPA compared to SHAM (p=0.0006) and OVX (p=0.007) (**Table 3**).

Percentages of 5-HETE and 12-HETE in bone marrow were significantly lower in the EPA and DHA groups compared to OVX and SHAM (5-HETE: DHA vs OVX p=0.042, EPA vs OVX p=0.029, EPA and DHA vs SHAM p<0.0001; 12-HETE: DHA vs OVX p=0.0007, EPA vs OVX p=0.0059, DHA vs SHAM p=0.0006, EPA vs SHAM p=0.0043) (**Table 3**).

Total amounts of all lipoxygenase–generated lipid mediators derived from DHA in bone marrow was not significantly different among groups. However the percentage of DHA pathway products (14-HDHA, 17-HDHA and 10,17-diHDHA NPD1/PD1 isomers) of the total bioactive LOX profile of products in bone marrow was significantly higher in the DHA supplemented group compared to SHAM (p=0.03) and OVX (p=0.0063) and tended to be higher than in the EPA group (p=0.069). The resolvin pathway marker, namely 17-HDHA, accounted for a significantly greater

proportion of total LOX product profiles in the DHA group than in the OVX ($p=0.008$) and EPA ($p=0.009$) groups. Conversely, the amounts of 15-HETE as a percentage of total LOX pathway products was significantly lower in the DHA group than in OVX ($p=0.003$), SHAM ($p=0.002$) and EPA groups ($p=0.004$) (**Table 3**).

Discussion

LOX families of bioactive mediators derived from AA, DHA and EPA namely lipoxins, resolvins and protectins, were present in rat bone marrow as identified by the presence of diagnostic ions in their corresponding MS-MS spectra. Ovariectomy resulted in a relative decrease in the proportion of LOX products derived from DHA compared to those from AA in bone marrow. Dietary supplementation with the ethyl ester of either DHA or EPA led to a decrease in the proportion of AA-derived LOX pathway products and an increase in the proportion of DHA or EPA-derived LOX products.

A relative deficiency of DHA in brain tissue has been suggested as a possible contributing factor to the pathogenesis of Alzheimer's disease [39]. Increased dietary intake of DHA substantially reduced amyloid production in a mouse model of Alzheimer's disease [39] and LOX-generated bioactive mediators derived from DHA have potent neuroprotective activity [40]. Dietary supplementation with DHA has also been shown to significantly reduce ovariectomy-induced bone mineral loss in rats, an animal model for postmenopausal osteoporosis [41]. That a relative deficiency in DHA-derived lipid mediators occurs post-ovariectomy and that this can be rectified by DHA supplementation may be of relevance to the observed bone- and brain- protective effects of DHA.

EPA ethyl ester supplementation resulted in only slight increases in the levels of LOX products obtained from DHA and conversely DHA ethyl ester supplementation resulted in only small increases in LOX pathway products of EPA in bone marrow. Although EPA can be further elongated and desaturated to form DHA and DHA can be retro-converted to EPA by endogenous enzymes, both processes are inefficient [42]. Earlier studies have reported only slight increases in tissue or blood levels of

EPA following DHA supplementation and of DHA following EPA supplementation [43, 44].

In the present study, ovariectomised animals were supplemented with EPA and DHA ethyl esters which clearly had an impact on increasing EPA and DHA LOX product profiles from our results. However, the requirement for cytoconversion of these esters to the free fatty acids to enable LOX-mediated generation of lipid mediators may have had an impact on the resultant profile of resolvin and non-resolvin pathway lipid mediators present in bone marrow. Both DHA and EPA ethyl ester supplementation significantly reduced the percentage of pro-inflammatory mediators present in bone marrow. Specifically, the proportion of HETES which are monohydroxy products of AA, was lower in bone marrow from DHA and EPA supplemented animals compared to ovariectomised controls. HETEs are implicated in a range of inflammatory disease processes including asthma [45] and psoriasis [46] and have been shown to inhibit the bone-forming ability of osteoblasts *in vitro* [47]. Possible therapeutic roles of n-3 LCPUFAs in the treatment of psoriasis, asthma and post-menopausal osteoporosis have previously been highlighted [41, 48-53].

As neither LXA4 nor LXB4 were present in any sample from the EPA group, EPA may inhibit the formation of 4-series lipoxins in bone marrow. Similarly, Ashton *et al* (1994) observed a significant reduction in the percentage of LXA4 produced by head kidney leucocytes of rainbow trout fed a n-3 LCPUFA-supplemented diet compared to trout fed a diet containing sunflower oil (devoid of n-3 LCPUFAs) [54]. LTB4 has been shown to promote neural stem cell proliferation and differentiation whereas LXA4 inhibits stem cell growth [33]. The lack of LXA4 in the EPA ethyl ester-supplemented group in the present study may have implications for the fate of stem cells within bone marrow.

The percentage of 18-HEPE, the precursor for E-series resolvins, of total LOX pathway products in bone marrow was significantly increased by EPA ethyl ester supplementation (Table 3). Likewise the percentage of 17-HDHA, an anti-inflammatory mediator [55] and a marker for D-series resolvins and protectins, was significantly increased by DHA ethyl ester supplementation. RvE1, RvD1 and PD1 were identified in some but not all bone marrow samples. This may be reflective of

their possible short half-life within marrow tissue and susceptibility to metabolic inactivation or could indicate that their biosynthesis in bone marrow is largely inducive rather than constitutive.

As resolvins and protectins exhibit potent bioactivity at nanomolar concentrations [56] in a range of cell types and tissues [15-17, 57-61], the presence of resolvins and protectins in bone marrow in the quantities observed in the present study is likely to be of physiological significance.

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CHAPTER 7

Mechanisms of action of DHA alone and in combination with oestrogenic compounds in MC3T3-E1/4 osteoblast-like cells

In Chapter 2 a beneficial effect of dietary supplementation with DHA on bone mass in ovariectomised rats was observed and in Chapter 5, a possible interaction between DHA and oestrogenic compounds was identified in vivo. The objective of the study described in this chapter was to elucidate the possible mechanism of action of DHA and to determine if an interaction between DHA and oestrogenic compounds occurs at the cellular level. As this is a large and complex field, the data presented in this chapter represent preliminary findings. It is intended that the results of the studies described herein be used to identify areas for future, more in-depth research.

Abstract

In the ovariectomised rat model for postmenopausal bone loss, dietary supplementation with DHA has been shown to significantly ameliorate ovariectomy-induced bone loss. Combined treatment with DHA and 17β-oestradiol has been associated with significantly greater bone–protective effects post-ovariectomy compared to either treatment alone. However no beneficial effect on bone mass was observed following combined treatment with DHA and either of the phytoestrogens genistein or daidzein post-ovariectomy in rats. The primary aim of the present study was to identify mechanisms of action of DHA in osteoblasts using the MC3T3-E1/4 cell line. Secondly, the possible interaction between DHA and oestrogenic compounds was explored and the effect of co-treatment with DHA and oestrogenic compounds on osteoblasts determined. Under conditions optimal for cell growth, DHA in concentrations of 2.5-10μg/ml had no observable effect on cell proliferation or differentiation. However, in MC3T3-E1/4 cells exposed to the pro-inflammatory cytokine TNF-α, DHA treatment was associated with a significantly higher cell number compared to non-DHA - treated TNF-α exposed cells. Cell number was significantly greater following combined treatment with 17β-oestradiol and DHA compared to either treatment alone. The mechanism did not involve altered PGE2 or TGF-β1 secretion as measured by ELISA or a change in nuclear membrane-bound levels of either oestrogen receptor (ER-α or ER-β) as measured by flow cytometry. Cell number was significantly lower in TNF-α exposed cells treated with DHA and genistein, but not daidzein, compared to TNF-α exposed controls. Mean PGE2 and TGF-β1 secretion per 10^5 cells were significantly higher than controls following treatment with genistein and DHA. As genistein is a tyrosine kinase inhibitor, results from the present study may indicate that DHA requires tyrosine kinase activity for its cytoprotective effects on osteoblasts following TNF-α exposure.

Introduction

Dietary intake of long chain polyunsaturated fatty acids (LCPUFAs) has been linked with a number of beneficial health effects in humans including a possible protective effect against postmenopausal bone loss. Various intervention studies in animal models for post-menopausal osteoporosis have demonstrated bone-sparing effects of n-3 LCPUFAs [1-3]. Specifically, docosahexaenoic acid (DHA, 22:6n-3) has been

shown to be particularly bone-protective (Chapter 2). Co-treatment with DHA and 17β-oestradiol has been shown to result in significantly higher bone mass in ovariectomised rats compared to either treatment alone (refer Chapter 5). However, co-treatment with DHA and either of the phytoestrogens daidzein and genistein may inhibit some of the effects of DHA on bone as bone mineral content (BMC) was found to be consistently, although not statistically significantly, lower in animals receiving both phytoestrogens and DHA compared to DHA alone. Determining how phytoestrogens and 17β-oestradiol interact with DHA may aid in elucidating the mechanism by which DHA acts on bone.

In the ovariectomised rat, DHA supplementation has been linked with increased periosteal circumference and greater overall bone area (Chapters 2 and 5); two observations which suggest DHA may promote new bone formation. However, the effects of DHA on osteoblasts are largely unknown. In the two studies which have explored the effects of DHA on osteoblast proliferation [4, 5], low concentrations of DHA appear to have no effect whereas high, and assumedly toxic, concentrations (>3 $\times 10^{-5}$M) inhibit cell growth [4, 5].

The objectives of the present study were to determine whether DHA treatment affects osteoblast growth *in vitro* and to provide insight into the mechanism by which phytoestrogens and 17β-oestradiol interact with DHA in bone.

Methods

Materials

Gamma-linolenic acid, docosahexaenoic acid, arachidonic acid, eicosapentaenoic acid, prostaglandin E2 (P0409) and β-Glycerophosphate (G-9891) were purchased from Sigma-Aldrich Co., New Zealand. Phenol red-free α-MEM (41061-029) and foetal calf serum were purchased from Invitrogen New Zealand Ltd. Ascorbic acid (Cat# 1831) was purchased from Merck Chemicals, Darmstadt, Germany. TNF-α (410-MT TNF-α/TNFSFIA) was purchased from R & D Systems, Minneapolis, USA. Axell Rabbit anti-bovine IgG, IgM and IgA was purchased from Accurate Chemical and Scientific Corp, New York, USA (catalogue number YMPS0113). Mouse IgG_{2a} γ isotype standard (HOPC-1) was purchased from Pharmingen (BD

Biosciences), San Jose, California, USA (catalogue number 03031D). Mouse anti-mouse Estrogen Receptor (ER)-α monoclonal antibody (catalogue number MA1-310) and rabbit anti-mouse ER-β polyclonal antibody (catalogue number PA1-311) were purchased from Affinity Bioreagents, Golden, Colorado, USA. Fluorescein (FITC)-conjugated AffiniPure F9ab′)$_2$ Fragment Goat anti-mouse IgG Fc$_\gamma$ fragment specific and R-phycoerythrein (PE-conjugated Affinipure F(ab′)$_2$ fragment goat anti-rabbit IgG (H+L) secondary antibodies were purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, Pennsylvania, USA).

Cell Culture Conditions

The MC3T3-E1/4 cell line is a pre-osteoblast cell line derived from murine (*Mus muscularis*) calvarial cells. Under appropriate growth conditions, MC3T3-E1/4 cells differentiate into mature osteoblasts and are capable of synthesising mineralised extracellular matrix [6]. As phenol red, a commonly used indicator in cell culture media, may have oestrogenic activity [7] and MC3T3-E1/4 cells are oestrogen-responsive [8], phenol red-free growth media was used for all experiments. MC3T3-E1/4 cells were plated in phenol red-free α-MEM with 10% foetal calf serum with and without treatments as indicated and incubated at 37°C in a humidified atmosphere of 95% air/ 5% CO_2. Cell density, number of replicate wells/treatment and specific culture conditions are as stated for each experiment. All experiments were independently replicated a minimum of three times.

Concentration of DHA to be used for in vitro experiments

As previous studies have shown little effect of DHA on osteoblast proliferation, EPA, GLA and AA treatments were included in order to provide additional information about the dose response of osteoblasts to LCPUFA treatment. MC3T3-E1/4 cells were plated at a density of 0.75×10^5 cells/ml in 96-well plates with 8 replicate wells per treatment. Cells were grown for 48hrs in phenol red-free α-MEM with 10% foetal calf serum and carrier (ethanol) or LCPUFA (EPA, GLA, AA or DHA) at concentrations of 2.5, 5.0, 10.0 or 20μg/ml. Concentration of ethanol in the cell culture media increased in both control and LCPUFA-treated cells as concentration of LCPUFA increased. Final ethanol concentration in cell culture media ranged from 0.0025% to 0.02%. For determination of cell differentiation, cells were seeded in 24-well plates at a concentration of 5.5×10^4 cells/ml. Cells were

cultured in phenol red-free α-MEM with 10% foetal calf serum, 10mM β-glycerophosphate and 50μg/ml ascorbic acid and carrier (ethanol) or LCPUFA (EPA, GLA, AA or DHA) at concentrations of 2.5, 5.0, 10.0 or 20μg/ml for 10 days. Media was changed every 2-3 days. Measurement of cell number and alkaline phosphatase activity were as described below.

Effect of DHA with and without genistein, daidzein or 17β-oestradiol on cell number in TNF-α exposed cells

MC3T3-E1/4 pre-osteoblast cells were cultured at a density of 7.5 x 10^4 cells/ml in 24-well plates with 4 replicate wells per treatment. Culture medium was phenol red-free α-MEM with 10% heat-inactivated foetal calf serum (FCS) and either no TNF-α (positive control), TNF-α (5ng/ml) alone or TNF-α (5ng/ml) with treatment. Treatments were DHA, genistein, daidzein, 17β-oestradiol, DHA + genistein, DHA + daidzein and DHA + 17β-oestradiol. Treatments were administered in the following concentrations: DHA 5μg/ml, genistein 10^{-6}M, daidzein 10^{-6}M and 17β-oestradiol 10^{-8}M. The carrier for DHA and 17β-oestradiol was ethanol and for genistein and daidzein, dimethylsulphoxide (DMSO). Final concentration of ethanol in cell culture media did not exceed 0.05% and concentration of DMSO in cell culture media was 0.01%. Cells were incubated for 6 days at 37°C in a humidified atmosphere of 95% air/ 5% CO_2. Media were changed following 48 hours of culture, to osteogenic media (phenol red-free α-MEM with 10% heat-inactivated FCS, 10mM β-glycerophosphate and 50μg/ml ascorbic acid) with and without TNF-α and treatments. Media were not subsequently changed during the remaining 4 days of culture to ensure that the concentrations of secreted proteins reached detectable levels. Each experiment was independently replicated at least three times.

Determination of cell number

Cell number was determined by crystal violet staining as previously described [9]. In short, after removal of media, cells were washed with PBS, fixed with 1% formaldehyde and incubated with 1% crystal violet for 60 minutes at 37°C. Following thorough washing, stain was extracted from cells with 0.2% Triton X-100 and absorbance read at 550nm using an ELx808 Ultra microplate reader (Bio-Tek Instruments Inc., Vermont, USA). Cell number was determined by normalising to the reading of a standard curve derived from a known number of cells per well.

Measurement of Alkaline Phosphatase Activity

Cells were washed with PBS, fixed with 1% formaldehyde and incubated with 300µl of alkaline phosphatase test reagent (0.05M p-nitrophenyl phosphate in TBS pH 9.5) for 1 hr at 37°C. Two samples (100µl) of test reagent from each well were transferred to a 96-well plate and absorbance read at 405nm using an ELx808 Ultra microplate reader (Bio-Tek Instruments Inc., Vermont, USA).

Determination of TGF-β1 and PGE2 in cell culture supernatant

Following the 6-day culture period, cell culture supernatant was removed and immediately frozen at -20°C until later analysis. Prior to analysis, supernatant was thawed and centrifuged at 2000rpm for 5 minutes to sediment any contaminating cells. Concentrations of TGF-β1 and PGE2 were determined by ELISA using commercially available ELISA kits as follows: Mouse/Rat/Porcine/Canine TGF beta-1 Quantikine ELISA kit (MB100B) and Prostaglandin E2 Parameter™ Assay kit (KGE004) supplied by R&D Systems, Minneapolis, USA.

Determination of nuclear membrane oestrogen receptor expression

Oestrogen receptor expression under a range of culture conditions was examined at culture day 8. All experiments were conducted using non-osteogenic media (phenol-red free α-MEM with 10% heat-inactivated FCS) and repeated using osteogenic media (phenol red-free α-MEM with 10% heat-inactivated FCS, 10mM β-glycerophosphate and 50µg/ml ascorbic acid). Cells (7.5 x 10^4 cells/ml) were incubated for 7 days with non-osteogenic or osteogenic media and either carrier (ethanol) or DHA (in concentrations of 5 and 10 µg/ml). In one set of experiments media were not changed for the 7-day incubation period. In a second set of experiments, media were changed every 3 days and in a third set the media was not changed throughout the culture period but DHA (5 or 10µg/ml) was added every 3 days. Following incubation, cells were washed five times with PBS, trypsinised and re-suspended in nuclear buffer (consisting of 5ml 1% sodium citrate, 50µl Triton-X 100 made up to a volume of 50mL with ddH_2O) at a concentration of 500,000 cells/200µl buffer. To 200µl of nuclei suspension, 5µl of primary antibody (0.5mg/ml) (either anti-ER-α, anti-ER-β or irrelevant control: Mouse IgG_{2a} γ isotype standard (HOPC-1) or Rabbit anti-bovine IgG, IgM and IgA) was added and nuclei incubated for 2 hours at 4°C. Nuclei were washed with PBS and 100µl of secondary

antibody (0.5-1.0mg/ml, FITC-conjugated Goat anti-mouse IgG or PE-conjugated goat anti-rabbit IgG) added. Nuclei were incubated for a further 2 hours at 4°C, washed with PBS and fixed with 400µl of 1% formaldehyde. Nuclei were analysed by flow cytometry using a FACSCalibur system and CellQuest software (BD Biosciences, San Jose, Ca, USA).

Statistical Analysis

Concentrations of PGE2 in cell culture media were $\log_{10}$-transformed to avoid heteroscadascity. All other data conformed to the requirements of the general linear model. Data were analysed by one-way ANOVA with post-hoc Tukey testing. A p-value of ≤ 0.05 was considered significant. Results are expressed as the mean ± SE of three independent experiments.

Results

Determination of DHA concentration for cell culture experiments

Cell number following treatment with EPA, DHA or GLA at concentrations of 2.5, 5, 10 or 20µg/ml was not significantly different from controls. Cell number was significantly higher than controls in cells treated with either 5 or 10µg/ml of AA ($p<0.0001$). Cell number was significantly lower in cells treated with 20µg/ml AA compared to cells treated with 10µg/ml AA ($p<0.0001$) (**Figure 1**). There was no significant effect of any LCPUFA treatment on mean alkaline phosphatase activity expressed on a per cell basis (data not shown). Unless otherwise stated, a concentration of 5µg/ml DHA was thus used in all subsequent experiments.

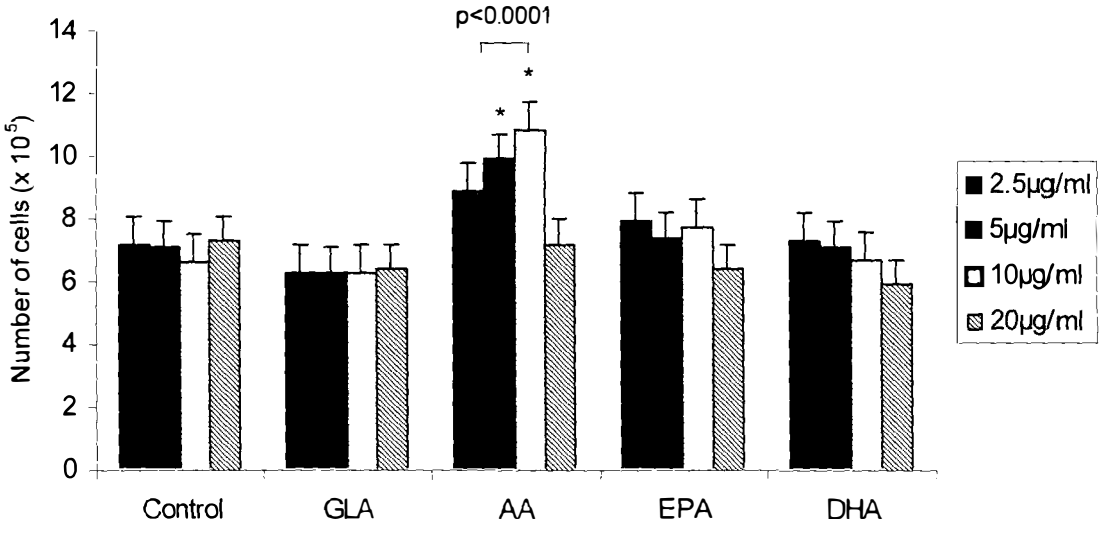

Figure 1 Effect of long chain polyunsaturated fatty acids (LCPUFA) on cell proliferation in MC3T3-E1/4 osteoblast-like cells. Cell number ($\times 10^5$) was determined following 48 hours of growth in 10% FCS/phenol-red free α-MEM with either the approproate amount of carrier (control cells) or GLA, AA, EPA or DHA at concentrations of 2.5, 5.0, 10.0 or 20.0 µg/ml. Carrier was ethanol. Final concentration of ethanol in cell culture media ranged from 0.0025% for the 2.5µg/ml dose of LCPUFA to 0.02% for 20µg/ml of LCPUFA. Cells were plated in 96-well plates at a density of 7.5×10^4 cells/ml with 8 replicate wells per treatment. Results shown are the mean and SE of three independent experiments. Cell count in treatments marked with * were significantly different from control at $p<0.05$.

Effect of DHA on cell number following exposure to TNF-α

Following 48 hours of culture, cell number was significantly lower in TNF-α–treated cells compared to non TNF-α treated controls ($p<0.0001$). Treatment with DHA had no significant effect on cell number after 48 hours of culture (data not shown). Following 6 days of culture, cell number was significantly lower in TNF-α –treated cells compared to non-TNF-α-treated controls ($p<0.0001$). Cell number was significantly higher in cells treated with both DHA and TNF-α compared to cells treated with TNF-α alone ($p=0.01$) (**Figure 2**). Next we sought to determine the effect of 17β-oestradiol (10^{-8}M), genistein or daidzein (10^{-6}M) alone, or in combination with DHA, on cell number in TNF-α exposed cells. Cell number was significantly lower in cells treated with both TNF-α and genistein than in cells treated with TNF-α alone ($p<0.0001$). Combined treatment with DHA, genistein and TNF-α was associated with significantly lower cell number than treatment with TNF-α + genistein ($p=0.0001$) or TNF-α + DHA ($p<0.0001$). Treatment of cells with TNF-α and daidzein had no statistically significant effect on cell number compared to

treatment with TNF-α alone. Cell number following treatment with TNF-α, daidzein and DHA was not significantly different from cells treated with TNF-α alone or DHA and TNF-α. In the absence of DHA, 17β-oestradiol had no statistically significant effect on cell number in TNF-α-exposed cells. However, cell number was significantly higher in cells treated with TNF-α, DHA and 17β-oestradiol compared to cells treated with TNF-α and DHA (p=0.02) (**Figure 2**).

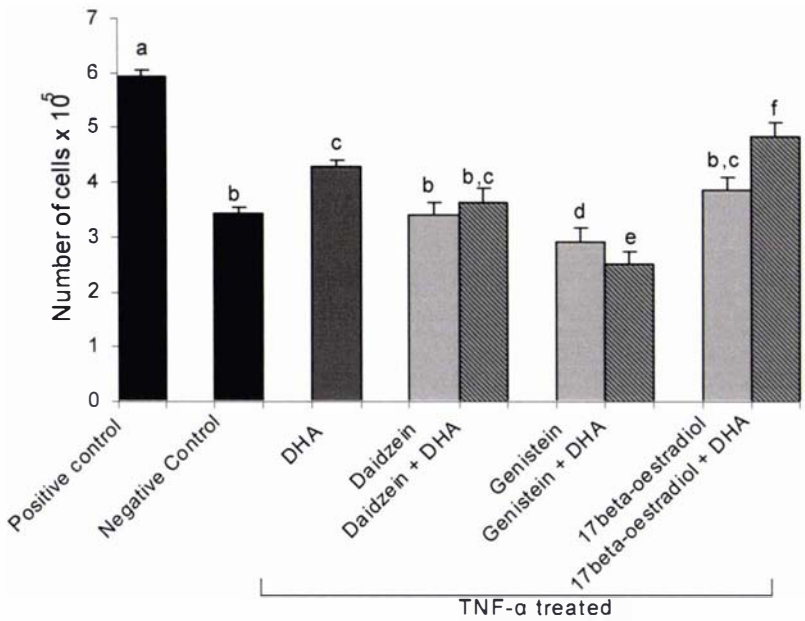

Figure 2 Effect of treatment with DHA and/or oestrogenic compounds on cell number in TNF-α – exposed MC3T3-E1/4 osteoblast-like cells. Cell number was measured by crystal violet staining (refer Methods) following 6-days of culture without TNF-α (positive control) or with TNF-α alone (negative control), or in conjunction with DHA (5μg/ml), daidzein (10^{-6}M), daidzein (10^{-6}M) + DHA (5μg/ml), genistein (10^{-6}M), genistein (10^{-6}M) + DHA (5μg/ml), 17β-oestradiol (10^{-8}M) or 17β-oestradiol (10^{-8}M) + DHA (5μg/ml). Results shown are the mean and SE of three independent experiments.

Effect of DHA and oestrogenic compounds on TGF-β1 secretion by TNF-α – exposed cells

Treatment of MC3T3-E1/4 cells with TNF-α had no statistically significant effect on mean TGF-β1 secretion per 10^5 cells compared to non-TNF-α treated controls (**Figure 3**).

A significant interaction between oestrogenic compounds and DHA treatment on mean TGF-β1 secretion per 10^5 cells was apparent (p=0.01). In the absence of oestrogenic compounds, treatment of cells with TNF-α and DHA had no significant

effect on mean TGF-β1 secretion per 10^5 cells. In the absence of DHA, treatment with genistein and TNF-α was associated with significantly higher mean TGF-β1 secretion per 10^5 cells ($p=0.0001$). Mean TGF-β1 secretion per 10^5 cells was significantly higher in cells treated with genistein, DHA and TNF-α compared to cells treated with DHA and TNF-α ($p<0.0001$) or genistein and TNF-α ($p=0.0004$). However, mean TGF-β1 secretion per 10^5 cells was not significantly different in cells treated with DHA and TNF-α compared to cells treated with TNF-α and either daidzein or 17β-oestradiol, with or without DHA (**Figure 3**).

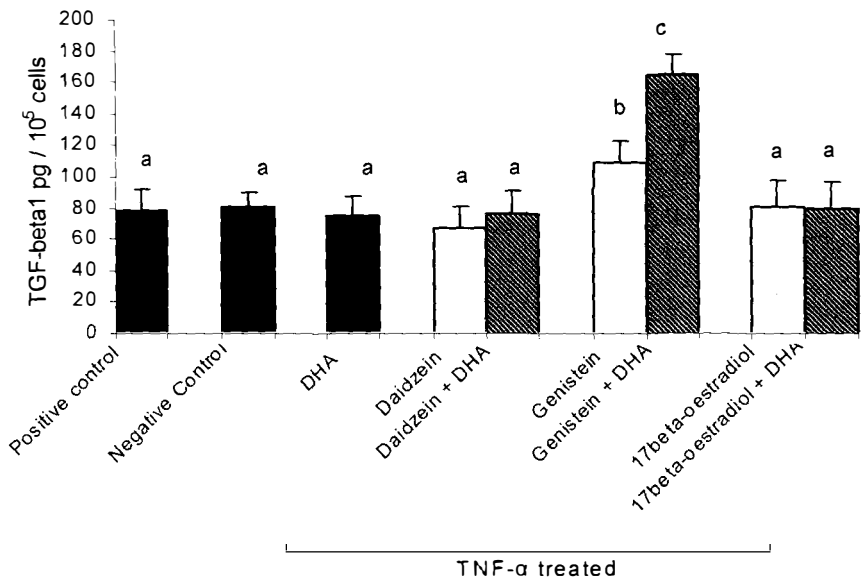

Figure 3 Effect of treatment with DHA and/or oestrogenic compounds on mean TGF-β1 secretion by TNF-α – exposed MC3T3-E1/4 osteoblast-like cells. TGF-β1 (pg / 10^5 cells) was measured by ELISA in cell supernatants following 6-days of culture without TNF-α (positive control) or with TNF-α alone (negative control) or in conjunction with DHA (5μg/ml), daidzein (10^{-6}M), daidzein (10^{-6}M) + DHA (5μg/ml), genistein (10^{-6}M), genistein (10^{-6}M) + DHA (5μg/ml), 17β-oestradiol (10^{-8}M) or 17β-oestradiol (10^{-8}M) + DHA (5μg/ml). Results shown are the mean and SE of three independent experiments.

Effect of DHA and oestrogenic compounds on PGE2 secretion by TNF-α – exposed cells

Treatment with TNF-α was associated with significantly greater mean PGE2 secretion per 10^5 cells compared to non-TNF-α treated cells ($p<0.0001$) (**Figure 4**). Again there was a significant interaction between treatment with oestrogenic compounds and DHA treatment on mean PGE2 secretion per 10^5 cells ($p=0.006$). In the absence of oestrogenic compounds, treatment with TNF-α and DHA had no significant effect on PGE2 secretion per 10^5 cells. In the absence of DHA, treatment

with TNF-α and daidzein was associated with a significant reduction in PGE2 secretion per 10^5 cells compared to TNF-α treatment alone (p=0.002). Conversely, treatment with TNF-α and genistein was associated with significantly higher PGE2 secretion per 10^5 cells than in TNF-α treated controls (p=0.008). Mean PGE2 secretion per 10^5 cells was not significantly different in cells treated with TNF-α and daidzein compared to cells treated with TNF-α, daidzein and DHA. Mean PGE2 secretion per 10^5 cells was significantly lower in cells treated with TNF-α, daidzein and DHA compared to cells treated with TNF-α and DHA (p=0.01). In contrast, mean PGE2 secretion per 10^5 cells was significantly higher in cells treated with TNF-α, genistein and DHA compared to cells treated with TNF-α and genistein (p=0.003) or TNF-α and DHA (p=0.0003). Mean PGE2 secretion per 10^5 cells was not significantly different in cells treated with TNF-α and 17β-oestradiol compared to treatment with TNF-α alone. Similarly, mean PGE2 secretion per 10^5 cells was not significantly different in cells treated with TNF-α, 17β-oestradiol and DHA compared to cells treated with TNF-α and DHA or TNF-α and 17β-oestradiol (**Figure 4**).

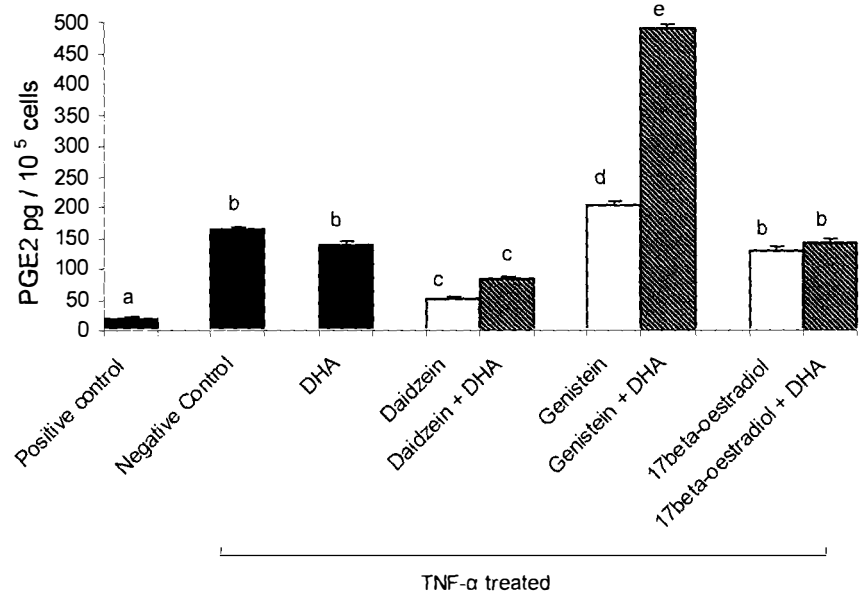

Figure 4 Effect of treatment with DHA and/or oestrogenic compounds on mean PGE2 secretion by TNF-α – exposed MC3T3-E1/4 osteoblast-like cells. PGE2 (pg / 10^5 cells) was measured by ELISA in cell supernatants following 6-days of culture without TNF-α (positive control) or with TNF-α alone or in conjunction with with DHA (5μg/ml), daidzein (10-6M), daidzein (10-6M)+DHA (5μg/ml), genistein (10-6M), genistein (10-6M)+DHA (5μg/ml), 17β-oestradiol (10-8M) or 17β-oestradiol (10-8M)+DHA (5μg/ml). Results shown are the mean and SE of three independent experiments.

Effect of DHA on nuclear membrane oestrogen receptor expression

At culture day 8 both ER-α and ER-β were detectable on MC3T3-E1/4 cell nuclei. There was considerable non-specific background binding of both secondary antibodies. DHA, at concentrations of 5μg/ml or 10μg/ml had no observable effect on ER-α or ER-β membrane expression whether added as a single dose with no media change, with media changes every 3 days, with DHA added but no media change every 3 days, using osteogenic media (α-MEM/10% FCS with ascorbic acid and β-glycerophosphate) or non-osteogenic media (α-MEM/10% FCS) with or without TNF-α (data not shown).

Discussion

Under conditions considered optimal for cell growth, DHA at concentrations of 2.5 – 10μg/ml appeared to have no effect on MC3T3-E1/4 cell proliferation or differentiation. In contrast, treatment with AA at concentrations of 5 or 10μg/ml increased MC3T3-E1/4 cell number but not mean alkaline phosphatase activity per cell. This suggests AA promoted proliferation but not differentiation of MC3T3-E1/4 cells. A growth-stimulatory effect of AA as well as of PGE2, a cyclooxygenase product of AA, has previously been demonstrated [10, 11]. In the present study, a concentration of 20μg/ml of DHA, EPA or AA was associated with reduced cell number. A 5-10μg/ml concentration range was thus chosen for assessing the effects of DHA on MC3T3-E1/4 cells.

One of the consequences of oestrogen deficiency is increased production of inflammatory cytokines such as tumour necrosis factor – α (TNF-α) [12] and this is believed to contribute to the pathogenesis of post-menopausal bone loss [13]. TNF-α exerts both cytotoxic and anti-proliferative effects in cells by inducing phosphorylation and dephosphorylation of specific proteins [14]. We sought to determine the effect of DHA on cell survival in osteoblasts exposed to pro-inflammatory TNF-α. As expected, in the present study cell number was significantly lower in TNF-α exposed cells compared to non-TNF-α exposed cells. Treatment of cells with DHA at the time of TNF-α challenge had no effect on cell survival in the first 48 hours of culture. However, by culture day 6, cell number was significantly higher in DHA-treated compared to non-DHA treated TNF-α exposed cells. DHA

has previously been shown to inhibit TNF-α activity and this is believed to contribute to the anti-atherogenic and anti-inflammatory effects of DHA in vascular cells [15, 16]. Whether DHA prevents apoptosis or promotes proliferation of TNF-α exposed osteoblasts remains to be determined.

As a significant interaction between DHA and oestrogenic compounds (genistein daidzein or 17β-oestradiol) has been observed when the compounds are administered to ovariectomised rats (Chapter 5), we investigated the effect of co-treatment with DHA and phytoestrogens or 17β-oestradiol on cell number following TNF-α exposure. In the absence of DHA, treatment with daidzein or 17β-oestradiol had no effect on cell number however treatment with genistein resulted in a significantly lower cell number compared to treatment with TNF-α alone. In fibroblasts, a cell morphologically similar to osteoblasts, genistein has been shown to synergistically potentiate the cytotoxic and anti-proliferative effects of TNF-α. This is believed to contribute to the anti-carcinogenic activity of genistein and the mechanism has been attributed to the tyrosine kinase inhibiting activity of genistein [14].

The protective effect of DHA on cell number was blocked by co-treatment with genistein but not daidzein. In a human kidney epithelial cell line DHA at concentrations of <10μM has been shown to stimulate cell proliferation under pro-tumourigenic conditions. Co-treatment with either of the tyrosine kinase inhibitors genistein or tyrphostin-47, prevented the growth stimulatory effect of DHA [17]. Although genistein is a tyrosine kinase inhibitor, daidzein lacks this ability. It is therefore possible that tyrosine kinase activity is required for the protective effect of DHA on cell number following TNF-α exposure in MC3T3-E1/4 cells.

TGF-β1 and PGE2 are two autacoids which potentially can mitigate TNF-α activity. TGF-β1 is a growth factor which inhibits the apoptotic effects of TNF-α [18]. PGE2 is an eicosanoid formed from AA by the action of cyclooxygenase (COX). PGE2 promotes proliferation of osteoblasts *in vivo* [19] and, to a lesser extent, *in vitro* [20] by activation of the MAP kinase signalling pathway [21]. Synthesis of PGE2 is increased in response to TNF-α [18].

In the present study DHA alone had no significant effect on mean TGF-β1 or PGE2 secretion by TNF-α-exposed MC3T3-E1/4 cells. Genistein significantly increased TGF-β1 and PGE2 secretion per 10^5 cells and the combination of genistein and DHA resulted in further enhancement of both TGF-β1 and PGE2 secretion. A stimulatory effect of genistein on TGF-β1 protein expression has previously been reported [22, 23]. As exogenous TGF-β1 promotes PGE2 synthesis in MC3T3-E1/4 cells [24, 25] and inhibition of endogenous TGF-β1 formation by serum deprivation is associated with reduced PGE2 synthesis [26], it is likely that the increased TGF-β1 secretion observed following genistein treatment in the present study contributed to the increased PGE2 secretion. Interestingly, treatment of serum-starved TNF-α exposed MC3T3-E1/4 cells with genistein, daidzein or 17β-oestradiol has been associated with reduced PGE2 secretion and increased cell number compared to cells treated with TNF-α alone [26]. Taken together, the results of the study involving serum-deprived cells and the results of the present study in which cells were provided with ample serum for growth may indicate that the three oestrogenic compounds can protect cells against some of the deleterious effects on cell number of serum deprivation but not of TNF-α.

The greater mean PGE2 secretion per 10^5 cells observed with genistein treatment could be expected to have led to increased cell number. However, tyrosine kinase activity is required for the growth stimulatory effect of PGE2 [18]. Therefore although PGE2 secretion was elevated with genistein treatment, the previously reported growth stimulatory effects of PGE2 [19] were possibly blocked by the tyrosine kinase inhibiting activity of genistein. This may partially explain the reduced cell number evident with genistein and genistein + DHA treatments in the present study. However as mean PGE2 secretion per 10^5 cells was significantly lower in daidzein-treated compared to DHA-treated or control cells, yet cell number was not significantly lower in daidzein treated cells, the influence of PGE2 on MC3T3-E1/4 cell number under these culture conditions may be relatively slight.

Combined treatment of MC3T3-E1/4 cells with 17β-oestradiol and DHA resulted in significantly higher cell number than treatment with 17β-oestradiol or DHA alone. Observations from the present study indicate the mechanism did not involve modulation of TGF-β1 or PGE2 secretion. As incorporation of DHA into cell

membranes can influence the expression of membrane-bound proteins, we investigated whether treatment of MC3T3-E1/4 cells with DHA alters nuclear membrane expression of the two types of oestrogen receptor, ER-α and ER-β. However under the range of culture conditions examined in the present study, there was no observable effect of DHA treatment on nuclear membrane expression of either oestrogen receptor.

In conclusion, DHA may mitigate the effect of TNF-α on cell number in MC3T3-E1/4 cells though the mechanism of action remains unclear. It appears that tyrosine kinase activity may be required for DHA activity. Co-treatment with 17β-oestradiol and DHA is more effective than either treatment alone in preserving cell number following TNF-α exposure. It is possible that preservation of osteoblast cell number following ovariectomy may have contributed to the observed beneficial effect of combined DHA and 17β-oestradiol treatment on bone mass post-ovariectomy in rats. Further work is required in order to determine the effect of DHA treatment, with and without 17β-oestradiol treatment, on osteoblast number following ovariectomy *in vivo*. There is a need to determine the signalling pathways which are influenced by DHA. Results from the present study suggest that tyrosine kinase receptor initiated pathways are likely candidates as DHA-responsive pathways.

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Discussion and Conclusions

Although a number of intervention studies have reported a beneficial effect of n-3 LCPUFAs on bone mass in ovariectomised animal models [1-3], only one intervention study has described a favourable effect of n-3 LCPUFA supplementation on bone mass in post-menopausal women [4]. Due to the limited evidence accrued to date, the bone-protective activity of n-3 LCPUFAs has yet to gain widespread acceptance in the medical community. National Health bodies in the US, Canada, Australia and New Zealand currently do not endorse a role for n-3 LCPUFAs in osteoporosis prevention [5-8]. More research is required in order to determine the type and dose of LCPUFA required for a bone protective effect and the mechanism involved.

The first objective of this thesis was to determine whether different LCPUFAs have differing bio-activities in bone. In Chapter 2, a direct comparison was made between the effects of GLA, DHA and EPA, the so-called "anti-inflammatory" lipids, on bone mass post-ovariectomy in rats. At the dose employed (0.5g LCPUFA/kg rat body weight/day), DHA alone exhibited significant bone-protective effects.

Previously, the bone-protective effect of n-3 LCPUFAs was attributed to their anti-inflammatory activity [9]. The finding that DHA, but not EPA, ameliorated ovariectomy-induced bone loss (reported in Chapter 2) suggests however that the mechanism of action is much more complex than simply an inhibition of inflammation. *In vitro* both EPA and DHA inhibited the PGE2-induced increase in membrane-bound RANKL expression in MC3T3-E1/4 cells (Chapter 3). However, there was no evidence of an anti-resorptive effect of either treatment *in vivo* as indicated by the lack of effect of DHA on reducing plasma CTX concentration and endosteal circumference compared to ovariectomised controls (Chapter 2). This may indicate that RANKL signalling is not the predominant pathway leading to the increased osteoclast number and activity which occurs post-ovariectomy. This is supported by a previous report which has shown that only small increases in RANKL are required to maximally stimulate osteoclast formation [10]. As a result it has been

suggested that RANKL-independent pathways have a major role in the increase in osteoclast formation evident post-menopause in women [10].

The greater bone area, larger periosteal circumference and increased plasma concentration of gamma-carboxylated osteocalcin in DHA-supplemented animals compared to ovariectomised controls reported in Chapters 2 and 5 suggest DHA may promote bone formation. The mechanism may involve the actions of vitamin D and IGF-1. The results presented in Chapter 2 showed elevated total 25-hydroxyvitamin D, but lowered 25-hydroxyvitamin D3 concentration and increased IGF-1 concentration in the plasma of DHA-supplemented rats. Whether these changes in plasma concentrations are a result of a change in the rate of synthesis or a change in the rate of turnover of IGF-1 and 25-hydroxyvitamin D remains to be determined.

Although DHA has also been shown to regulate gene expression of various cytochrome P450-dependent hydroxylases [11] such as cyp26 which is involved in retinoic acid metabolism [12], the possible effect of DHA on cytochrome P450 hydroxylases responsible for vitamin D metabolism has yet to be explored. As different 25-hydroxylases have differing specificities for vitamin D2 and D3 [13], modulation of the activity of the various hydroxylases may result in a change in the relative balance of hydroxylated metabolites of vitamin D2 and D3 such that vitamin D2 predominates. Other studies have reported that vitamin D2 is less potent than vitamin D3 in promoting calcium release from bone [14] and inducing hypocalcaemia [15] but more effective at promoting bone collagen synthesis [14]. The ultimate effect of DHA treatment on the activity of the various hydroxylases involved in vitamin D metabolism requires further research. Interestingly, specific metabolites of both vitamin D and retinoids have been shown to modify gamma-carboxylation of osteocalcin in human osteoblasts [16] and to influence gamma-carboxylase activity in rats, independent of the actions of vitamin K [17]. DHA-mediated modification of vitamin D and/or retinoid metabolism may have contributed to the increased plasma concentration of gamma-carboxylated osteocalcin reported in Chapter 5.

IGF-1 is produced by a range of cell types including osteoblasts. Its expression is induced by various hormones and cytokines such as PTH, leptin PGE2, TGF-β, and FGF2 (Fibroblast Growth Factor 2) [18, 19]. *In vivo* we found no evidence of an

increase in PTH level following DHA supplementation (Chapter 2) and *in vitro*, osteoblast secretion of TGF-β1 and PGE2 were not increased following DHA treatment in TNF-α-exposed cells (Chapter 7). The n-3 LCPUFAs have previously been shown to inhibit leptin expression [20] however the effect of DHA on FGF2 expression has yet to be determined and this warrants further investigation. IGF-1 promotes proliferation and differentiation of osteoblasts by binding to its membrane-bound receptor. The cytoplasmic domain of the IGF-1 receptor has tyrosine kinase activity and this activity is essential for IGF-1 signalling [21]. In Chapter 7, the tyrosine kinase inhibiting activity of genistein was proposed as a possible contributor to the mechanism by which genistein prevented the DHA-induced promotion of cell number following TNF-α exposure in MC3T3-E1/4 osteoblast-like cells. Inhibition of IGF-1 signalling may have contributed to the reduced cell number evident in osteoblasts treated with genistein and DHA compared to DHA alone.

In the study presented in Chapter 5, an interaction between DHA and oestrogenic compounds was observed such that RBC fatty acid composition, plasma IL-6 concentration and BMC differed depending on the type of oestrogenic compound administered in conjunction with DHA. Although there was an additive or synergistic effect of DHA and 17β-oestradiol on BMC, there was no beneficial effect of combined phytoestrogen and DHA treatment on bone mass (Chapter 5). Incorporation of DHA into membranes alters the amount and type of membrane-bound proteins [22, 23]. As phytoestrogens predominately interact with oestrogen receptor β (ERβ) whereas 17β-oestradiol interacts with both ERα and ERβ, we sought to determine whether DHA alters the relative levels of the two oestrogen receptors, ERα and ERβ, in the nuclear membrane of osteoblasts (Chapter 7). Under the study conditions employed in Chapter 7, we observed no effect of DHA on ERα or ERβ expression in the nuclear membrane. It would be of interest to determine if DHA alters oestrogen receptor activation (particularly activation of ERα) and/or interacts with ERα signalling.

A major question still remaining is why DHA fails to exert any observable effect on osteoblasts under conditions optimal for cell growth (Chapter 7). Although there is little published literature on the effects of DHA in bone cells, there is currently considerable research interest into the involvement of DHA and EPA in soft tissue

repair. In many ways the process of bone remodelling, which is initiated in response to micro-damage to bone tissue, mimics the process of soft tissue repair which is instigated in response to injury or infection. Pro-inflammatory mediators are released as an early response to damage in soft and mineralised tissue. It is now known that other lipid mediators, such as the lipoxins, are endogenous "stop" signals which prevent further pro-inflammatory mediator production within soft tissue. A third set of lipid mediators consisting of the resolvins and docosatrienes inhibit the effects of pro-inflammatory mediators and trigger the resolution of inflammation and ultimate completion of the soft tissue repair process. Whereas pro-inflammatory mediators and lipoxins are derived from n-6 LCPUFAs, resolvins and docosatrienes are derived from EPA and DHA. Resolvins and docosatrienes are present in bone marrow in relatively high concentrations (Chapter 6). As seen in Chapter 6, ovariectomy alters the balance between pro-inflammatory AA-derived and pro-resolving DHA-derived lipid mediators in bone marrow and this balance is somewhat restored by DHA supplementation. Given the parallels between the regulatory processes governing soft tissue repair and mineralised tissue remodelling, it is possible that resolvins and docosatrienes derived from DHA, rather than DHA itself, are responsible for at least some of the bone-protective effects of DHA. The mechanism by which resolvin and docosatriene synthesis is induced is currently unknown however biosynthesis is known to be initiated as an early response to infection or injury. If resolvins and docosatrienes, rather than DHA, are bioactive in osteoblasts, DHA treatment would have no effect on osteoblasts except under pro-inflammatory conditions. This may explain why DHA treatment only elicited a response in osteoblasts when co-administered with the pro-inflammatory cytokine TNF-α, a known stimulator of lipoxygenase activity [24]. The role of lipid mediators derived from DHA on bone cell function remains to be explored.

Recommendations for Future Research

- Determining the signal transduction pathway(s) employed by DHA in bone cells is a critical next step to understanding the mechanism by which DHA acts in bone. Observations from the present thesis suggest that the following two pathways are possible candidates as DHA-responsive pathways:
  - the Ras/Raf-1/MAPK pathway, a pathway initiated by IGF-1.

- the cAMP pathway. This is a pathway activated by PGE2 which ultimately leads to up-regulation of RANKL expression. DHA has previously been shown to reduce cAMP signalling in non-bone cells [25, 26].

- The role of docosatrienes and resolvins derived from both DHA and EPA, both on bone cell formation and activity *in vitro* and on bone mass in growing and ovariectomised animals, requires investigation.

- Whether DHA (or lipid mediators formed from DHA) are bioactive when present within membranes in bone cells or whether their release from bone cell membranes is required for bioactivity needs to be determined.

- Further *in vivo* studies to establish the optimal dose of DHA for bone mass preservation post-ovariectomy in animal models are required. It would also be of interest to determine whether the type or amount of other lipids in the diet (eg n-6 LCPUFAs) influences DHA bioactivity.

- The effectiveness of DHA supplementation in the prevention of post-menopausal osteoporosis in women needs to be evaluated. If proven efficacious, a recommended dietary intake of DHA to aid in osteoporosis prevention needs to be established.

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Appendix 1

Phytoestrogens and Bone – Summary of Studies in Humans

Epidemiological Studies						
Phytoestrogen	**Model**	**Mean soy intake**	**Mean calcium intake**	**Outcome**		**Ref**
				Positive	**Nil/Negative**	
Total soy	Chinese women	25.4mg isoflavones/day	657mg/day	BMD LS & Wards triangle Lower PTH, Osteocalcin & urinary NTX	No effect BMD femur	[1]
Genistein, daidzein, Equol	Post-menopausal Korean women	Not reported Urinary excretion in μmol/day was genistein 1.09, daidzein 2.59 & equol 0.93	574-650mg/day		N/S LS, femoral neck, Ward's triangle BMD	[2]
Enterolactone	Post-menopausal Korean women	Not reported. Urinary excretion was 1.46μmol/day	574-650mg/day	BMD – LS, Fem Neck, Ward's triangle		[2]
Soy protein	Post-menopausal Japanese women	12.6g soy protein/day		LSBMD Lower DPYD excretion		[3]
Genistein & daidzein	Post-menopausal Japanese women	18.4g/day Highest tertile 22.5g/day	623mg/day	LS and femoral neck BMD. Both were significantly higher in highest tertile of gen & dai intake.		[4]
Genistein & daidzein	Pre & Post-menopausal Chinese women	8.8mg/day	677mg/day		No association LS and femoral neck BMD	[4]
Soy protein	Early postmenopausal Chinese women	Highest quartile: 19.4g soy protein /day Range 9.6-79.6g/day	Not reported	Increased BMD in women > 4 years post-menopause with increasing quartile of soy intake	No effect of soy intake on BMD in women <4 yrs post-menopause	[5]
Total genistein intake	Postmenopausal women	Highest tertile >1000μg genistein/day	Not reported	Lower urinary NTX excretion in those with highest intakes compared to those with no isoflavone intake	No effect BAP, PYR, BMD (femoral neck, trochanter, total hip, total spine.)	[6]

Intervention Studies								
Phytoestrogen	**Study population**	**Study duration**	**Mode**	**Soy Protein present?**	**Calcium**	**Outcome**		**Ref**
						Positive	**Nil/Negative**	
50mg isoflavones/day	Adolescent males	6 weeks	Tablet	Not reported	Not reported		No effect on BAP, PYR	[7]
52mg isoflavones/day	Premenopausal (24yrs old) caucasian women	One menstrual cycle	Food	Yes	1168mg/day		Increased ratio of CTX:osteocalcin	[8]
64mg isoflavone aglycones/day	Premenopausal women	Approx 93 days	Food	Yes	Baseline: 848mg/day Trial: 1494mg/day	Increased IGF1 & IGFBP3 at certain stages of menstrual cycle	No effect CTX or BAP Increased Dpyd excretion	[9]
90mg soy isoflavones/day	Premenopausal women 21-25 yrs old	12 months	Food	Yes	Baseline: 110mg/day Trial: 830mg Ca/day		No effect on BMC or BMD at lumbar spine, greater trochanter (femur) or Wards triangle (femur)	[10]
128mg isoflavone aglycones/day	Premenopausal women	Approx 93 days	Food	Yes	Baseline: 848mg/day Trial: 592mg/day		No effect CTX, IGF1, IGFBP3 or BAP Increased Dpyd excretion at certain stages of menstrual cycle	[9]
Low isoflavone (<4mg/day)	Early post-menopausal women	9 months	Food	Yes	Not reported	BMD trochanter		[11]
4.4mg soy isoflavones	Early postmenopausal women	24 weeks	Food	Yes	Baseline: 450mg/day Trial: 810mg Ca/		No effect on NTX or BAP No effect on LSBMD or LSBMC once covariates accounted for	[12]
Soy isoflavones 30.9mg	Early postmenopausal	4 weeks	Tablet	Yes	Not reported	Decreased PYD & DPYD excretion cf baseline	No effect osteocalcin	[13]
37.3mg/day soy isoflavones	Early post-menopausal Japanese living in Brazil	10 weeks	Food	Yes	Not reported	Decreased PYD & DPYD excretion	N/S bone stiffness	[14]

Intervention Studies								
Phytoestrogen	**Study population**	**Study duration**	**Mode**	**Soy Protein present?**	**Calcium**	**Outcome**		**Ref**
						Positive	**Nil/Negative**	
40mg soy isoflavones/day	Chinese early postmenopausal women	1 year	Tablet	Not reported	Baseline : not reported Trial: 1196mg/day 500mg Ca & 125IU vit D provided as supplement		No effect on BMC or BMD	[15]
41.9mg soy isoflavones/day	Early postmenopausal women	16 weeks	Food	Yes	Not reported		No effect BAP or DPYD	[16]
47mg isoflavones/day	Early postmenopausal women	6 months	Food	Yes	Not reported	Trend for osteocalcin to increase	No effect forearm BMD or NTX or hydroxyproline excretion	[17]
Genistein 54mg/day	Early postmenopausal women	1 year	Tablet	No	Baseline: 889-920mg/day Trial: not stated but those with initial intakes below 500mg/day were advised to increase intake	Reduced PYR & DPYR excretion Increased osteocalcin & B-ALP Increased BMD femoral neck & lumbar spine		[18]
High isoflavone (54 or 90mg/day)	Early post-menopausal women	9 months	Food	Yes	Not reported		No effect BMD	[11]
80mg soy isoflavones/day	Chinese early postmenopausal women	1 year	Tablet	Not reported	Baseline: not reported Trial: 1127mg/day 500mg Ca & 125IU vit D provided as supplement	Increased BMC at total hip & trochanter in women with low initial BMC		[15]

Intervention Studies								
Phytoestrogen	**Study population**	**Study duration**	**Mode**	**Soy Protein present?**	**Calcium**	**Outcome**		**Ref**
						Positive	**Nil/Negative**	
80.4mg soy isoflavones	Early postmenopausal women	24 weeks	Food	Yes	Baseline: 450mg/day Trial: 810mg/day provided in supplements & soy foods.	Prevented LSBMC and BMD loss	No effect on NTX or BAP	[12]
90mg pure genistein/day	Early postmenopausal women	6 weeks	Tablet	No	Not reported		N/S effect on osteocalcin or CTX	[19]
0mg/day	White, US postmenopausal women	1 month (4 weeks)	Food	Yes	Ca approx 1000mg/day		No effect Ca balance or biochemical markers of bone metabolism	[20]
22.4mg soy isoflavones/day	Postmenopausal women – Japanese?	12 weeks	Food	Yes	Soy food contained 51.6mg Ca/day	BAP increased TRAP decreased	No change metacarpal BMD	[21]
33.5mg soy isoflavones/day	Postmenopausal women – Japanese?	12 weeks	Food	Yes	Soy food contained 60.4mg Ca /day	Increased metacarpal BMD	No effect BAP or TRAP	[21]
40mg isoflavones/day	Postmenopausal, climacteric Japanese women	8 weeks	Food	Not reported	Not reported	Decreased Dpyd excretion	No effect BMD or osteocalcin	[22]
43.5mg total/day consisting of: 26mg biochanin A, 16mg formonectin, 1mg genistein and 0.5mg daidzein	Pre-, peri- and post-menopausal women (68-69% post-menopausal)	12 months (52 weeks)	Tablet	No	1007-1013mg/day Vitamin D 3.19-3.37µg/day	Trend for increased PINP (N/S) Reduced BMC and BMD loss at LS	No significant effect on hip BMC or BMD	[23]

Intervention Studies								
Phytoestrogen	**Study population**	**Study duration**	**Mode**	**Soy Protein present?**	**Calcium**	**Outcome**		**Ref**
						Positive	**Nil/Negative**	
56mg soy isoflavones/day	Hypercholesterolemic postmenopausal women	26 weeks	Food	Yes	Not reported		No effect BMC or BMD - any site	[24]
57mg soy isoflavones (aglycones)/day	Postmenopausal women	7 weeks	Food	Yes	746mg/day		No effect urinary hydroxyproline or N-telopeptide. No effect serum B-ALP, osteocalcin. No effect calcium retention	[25]
60mg/day	Postmenopausal women	12 weeks	Food	Yes	Not reported	Increased serum osteocalcin Decreased NTX excretion		[26]
65mg isoflavone aglycones/day	Postmenopausal women	93 days (13 weeks)	Food	Yes	Baseline: 945mg/day Trial: 1047mg/day Vit D intake also increased significantly	Decreased BAP	No effect CTX, Dpyd, IGF1 or IGFBP3	[9]
65mg/day soy isoflavones	White, US postmenopausal women	1 month (4 weeks)	Food	Yes	Ca approx 1000mg/day		No effect Ca balance or biochemical markers of bone metabolism	[20]
76mg soy isoflavones/day	Postmenopausal Caucasian (Danish)	2 years (104 weeks)	Food	Yes	Not reported	LSBMC & BMD increased	No change femoral neck BMC or BMD N/S changes in serum PINP and ICTP	[27]
84mg isoflavones/day	Postmenopausal Chinese women	24 weeks	Tablet	Not reported	Not reported	Decreased Dpyd excretion	N/S BMD LS, femur neck, Ward's triangle No effect osteocalcin or BAP	[28]

Intervention Studies								
Phytoestrogen	**Study population**	**Study duration**	**Mode**	**Soy Protein present?**	**Calcium**	**Outcome**		**Ref**
						Positive	**Nil/Negative**	
90mg soy isoflavones/day	Hypercholesterolemic postmenopausal women	26 weeks	Food	Yes	Not reported	Increased LSBMC & LSBMD	No effect on BMC/BMD at other skeletal sites	[24]
99mg soy isoflavones/day	Postmenopausal women Netherlands	12 months (52 weeks)	Food	Yes	Baseline: 1623mg/day Trial: 1212mg/day		No effect BMD –femur & LS	[29]
100mg isoflavones/day	Postmenopausal women	2 years (104 weeks)	Food	Yes	1200-1500mg Ca/day	Maintained BMC & BMD at LS		[30]
110mg soy isoflavone/day	Postmenopausal women	6 months (26 weeks)	Tablet	Not reported	Baseline: 825mg/day Trial: 1200mg/day	Decreased type 1 collagen α_1 chain helical peptide excretion Greater BMD L2 & L3.	N/S total spine & hip BMC & BMD	[31]
114mg isoflavonoids/day	Postmenopausal women with a history of breast cancer	3 months (13 weeks)	Tablet	Not reported	300-1000mg/day	Reduced Pyr & Dpyr excretion	No effect BAP, PINP, PICP	[32]
118mg isoflavones/day	Postmenopausal women	3 months (13 weeks)	Food	Yes	Baseline: 1358-1379mg/day Trial: not reported but soy milk supplement & placebo also contained Ca		No effect PYD & DPYD	[33]
126mg isoflavones/day	Postmenopausal Chinese women	24 weeks	Tablet	Not reported	Not reported	Decreased Dpyd excretion Trend for BMD to be higher with high dose (N/S)	No effect osteocalcin or BAP	[28]

Intervention Studies								
Phytoestrogen	**Study population**	**Study duration**	**Mode**	**Soy Protein present?**	**Calcium**	**Outcome**		**Ref**
						Positive	**Nil/Negative**	
132mg isoflavone aglycones/day	Postmenopausal women	93 days (13 weeks)	Food	Yes	Baseline: 945mg/day Trial: 1094mg/day Vit D intake also increased significantly	Decreased BAP	No effect CTX	[9]
1500mg nijiru (soybean extract)/day (tablet) Isoflavone content not reported	Adult men & women consuming a low vitamin K diet during study period No control group consuming a vitamin K adequate diet	60 days	Food	Not reported	Not reported	Reduction in serum calcium in women (but not men). Reduction in serum inorganic phosphorus & increase in γ-carboxylated osteocalcin in both men & women.		[34]

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Appendix 2

Phytoestrogens and Bone – Summary of Studies in Animals

Phyto source	Dose	Mode	Model	Study length	Soy protein present?	Dietary calcium content	Outcome				Ref
							Calcium Balance	Biochemical Markers	BMC/ BMD	Other	
Growing OVX rat											
Soy protein isolate Ethanol extraction of isoflavones	Approx 0.84mg/day (gen + dai) Equivalent to 4mg/kg body wt at trial commencement & 3mg/kg body weight at trial completion	Diet	OVX rat Sprague Dawley 95 days (3mo)	35 days (5 weeks)	Yes	0.4% of diet		No effect serum ALP, urinary hydroxyproline	No effect femoral BMD	No effect calcitriol or calcidiol	[1]
Soy protein isolate	Approx 8.39mg/day (gen + dai) Equivalent to 39.8mg/kg body wt at trial commencement & 28.4mg/kg body weight at trial completion	Diet	OVX rat Sprague Dawley 95 days (3mo)	35 days (5 weeks)	Yes	0.4% of diet		No effect serum ALP, urinary hydroxyproline	Greater femoral BMD	No effect calcitriol or calcidiol	[1]
Soy protein isolate	Approx 8.39mg/day (gen + dai) Animal body weight not reported	Diet	OVX rat Sprague Dawley 95 days (3mo)	65 days (supplementation commenced 35 days following OVX)	Yes	Not reported		No effect ALP, acid phosphatase	No effect femoral or 4th lumbar vertebrae BMD	No effect calcidiol or calcitriol	[2]

Phyto source	Dose	Mode	Model	Study length	Soy protein present?	Dietary calcium content	Outcome				Ref
							Calcium Balance	Biochemical Markers	BMC/ BMD	Other	
Soy protein isolate. Ethanol extraction of isoflavones	Approx 0.84mg/day (gen + dai) Animal body weight not reported	Diet	OVX rat Sprague Dawley 95 days (3mo)	65 days (supplementation commenced 35 days following OVX)	Yes	Not reported		No effect ALP, acid phosphatase	No effect femoral or 4th lumbar vertebrae BMD	No effect calcidiol or calcitriol	[2]
Isoflavones Source & composition not reported	Approx 0.96mg per rat per day Equivalent to 3.5mg/kg body weight per day	Diet	OVX rat Sprague Dawley 90 days (3mo)	40 days	Not reported	0.4% of diet	No effect urinary Ca or Mg excretion	No effect serum ALP or TRAP	No effect on 4th lumbar vertebral, tibial or femoral BMD		[3]
Isoflavones Source & composition not reported	Approx 1.92mg per rat per day Equivalent to 7mg/kg body weight per day	Diet	OVX rat Sprague Dawley 90 days (3mo)	40 days	Not reported	0.4% of diet	No effect urinary Ca or Mg excretion	No effect serum ALP or TRAP	No effect on 4th lumbar vertebral, tibial or femoral BMD		[3]
Soy protein isolate	Not reported	Diet	OVX rat Sprague Dawley 90 days (3mo)	35 days	Yes	0.4% of diet	Prevented OVX-induced reduction in Ca transport in duodenal & colonic cells			No effect IGF-1, calcidiol or calcitriol	[4]

Phyto source	Dose	Mode	Model	Study length	Soy protein present?	Dietary calcium content	Outcome				Ref
							Calcium Balance	Biochemical Markers	BMC/ BMD	Other	
Soy protein isolate	Not reported	Diet	OVX rat Sprague Dawley 90 days (3mo)	35 days	Yes	0.4% of diet	No effect intestinal Ca transport			No effect calcidiol, calcitriol, IGF-1	[4]
Genistein	1µg/g body weight (1mg/kg body weight)	s.c.	OVX rat Sprague Dawley 60 days (2mo)	21 days	No	0.6% of diet			No effect tibial BMD		[5]
Genistein	5µg/g body weight (5mg/kg body weight)	s.c.	OVX rat Sprague Dawley 60 days (2mo)	21 days	No	0.6% of diet		No effect DPYD Increased serum osteocalcin	No effect tibial BMD		[5]
Isoflavones	6.25g/kg diet Approx 50mg/kg body weight/day	Diet	OVX rat Sprague Dawley 12 weeks (3mo)	16 weeks	No	0.4% of diet		No change ALP or TRAP.	No change tibia BMD No change bone Ca content – femur & tibia Increased femoral & LS vertebral BMD Higher bone ash content		[6]

Phyto source	Dose	Mode	Model	Study length	Soy protein present?	Dietary calcium content	Outcome				Ref
							Calcium Balance	Biochemical Markers	BMC/ BMD	Other	
Growing OVX rat LOW CALCIUM DIET											
Isoflavones	80ppm in diet 0.08g/kg diet Approx intake: 4mg/kg body weight/day	Diet	OVX rat Sprague Dawley 9weeks (2mo)	6 weeks	No	0.1% of diet		No change serum ALP, TRAP, urinary hydroxyproline	Increased Ca content of femur & LS. Increased LS dry weight	No effect femur breaking force.	[7]
Isoflavones	160ppm in diet 0.16g/kg diet Approx intake: 8mg/kg body weight/day	Diet	OVX rat Sprague Dawley 9weeks (2mo)	6 weeks	No	0.1% of diet		No change serum ALP, TRAP, urinary hydroxyproline,	Increased LS dry weight No effect Ca content of femur or LS	No effect femur breaking force,	[7]
Growing OVX mice											
Genistein	0.4mg/day	s.c	OVX mice ddy 8weeks (2mo)	4 weeks	No	0.4% of diet			Increased femoral BMD		[8]
Genistein	0.7mg/day	s.c.	OVX mice ddy 8weeks (2mo)	4 weeks	No	0.4% of diet			Femoral BMD N/S compared to sham		[8]
Daidzein	200mg/kg diet Approx: 22mg/kg body weight/day	Diet	OVX mice C57, C3H, CD-1 & Swiss Webster 8 weeks (2mo)	3 weeks	No	Not reported			No effect LS or femur BMC, BMD	No effect peak load	[9]

Phyto source	Dose	Mode	Model	Study length	Soy protein present?	Dietary calcium content	Outcome				Ref
							Calcium Balance	Biochemical Markers	BMC/ BMD	Other	
Isoflavones	160mg isoflavone conjugates/kg body weight/day	Diet	OVX mice C57, C3H, CD-1 & Swiss Webster 8 weeks (2mo)	6 weeks	Not reported	Not reported			Increased whole body, femoral & LS BMD		[10]
Mature OVX rat											
Isoflavone-enriched soy protein isolate	0.4mg isoflavones/g diet	Diet	OVX rat Sprague Dawley 6mo	8 weeks	Yes	0.54%	Lower urinary Ca excretion		No effect on femoral or whole body BMD		[11]
Isoflavone-enriched soy protein isolate	0.2mg isoflavones/g diet	Diet	OVX rat Sprague Dawley 6mo	8 weeks	Yes	0.54% of diet	Lower urinary Ca excretion		No effect on femoral or whole body BMD		[11]
Soy protein isolate	Negligible	Diet	OVX rat Sprague Dawley 6mo	8 weeks	Yes	0.54% of diet	Lower urinary Ca excretion		No effect on femoral or whole body BMD		[11]
Isoflavone extract	0.3mg isoflavones/g diet	Diet	OVX rat Sprague Dawley 6mo	8 weeks	No	0.54% of diet	Lower urinary Ca excretion		No effect on femoral or whole body BMD		[11]

Phyto source	Dose	Mode	Model	Study length	Soy protein present?	Dietary calcium content	Outcome				Ref
							Calcium Balance	Biochemical Markers	BMC/ BMD	Other	
Isoflavone extract	0.8mg isoflavones/g diet	Diet	OVX rat Sprague Dawley 6mo	8 weeks	No	0.54% of diet	Lower urinary Ca excretion		No effect on femoral or whole body BMD		[11]
Isoflavones	20mg/kg body weight/day	Diet	OVX rat Wistar 195 day (6.5mo)	84 days starting 80 days post-OVX	No	0.24% of diet			No effect BMD or cancellous BA		[12]
Isoflavones	40mg/kg body weight/day	Diet	OVX rat Wistar 195 day (6.5mo)	84 days starting 80 days post-OVX	No	0.24% of diet		Reduced plasma osteocalcin & urinary DPYDs	No effect BMD or cancellous BA		[12]
Isoflavones	80mg/kg body weight/day	Diet	OVX rat Wistar 195 day (6.5mo)	84 days starting 80 days post-OVX	No	0.24% of diet			No effect BMD or cancellous BA		[12]
Isoflavones	20mg/kg body weight/day	Diet	OVX rat Wistar 195 day (6.5mo)	91 days	No	0.24% of diet		Prevention of initial OVX-induced increase in plasma osteocalcin	No effect metaphyseal BMD Higher total femoral & diaphyseal BMD	Increased femoral strength	[13]
Isoflavones	40mg/kg body weight/day	Diet	OVX rat Wistar 195 day (6.5mo)	91 days	No	0.24% of diet		Prevention of initial OVX-induced increase in plasma osteocalcin	Higher total femoral, metaphyseal & diaphyseal BMD	Increased femoral strength	[13]

Phyto source	Dose	Mode	Model	Study length	Soy protein present?	Dietary calcium content	Outcome				Ref
							Calcium Balance	Biochemical Markers	BMC/ BMD	Other	
Isoflavones	80mg/kg body weight/day	Diet	OVX rat Wistar 195 day (6.5mo)	91 days	No.	0.24% of diet		Prevention of initial OVX-induced increase in plasma osteocalcin	Higher total femoral & metaphyseal BMD	Increased uterine weight Increased femoral strength	[13]
Aged OVX rat											
Isolated soy protein	140g/kg diet isolated soy protein Isoflavone content not reported	Diet	OVX rat Sprague Dawley 11mo (retired breeders)	3 months	Yes	Not reported			Greater femoral BMD		[14]
Genistein	10mg/kg body weight/day	Diet	OVX rat Wistar 12mo	90 days	No	0.23% of diet			No effect LS, total femur & metaphyseal BMD Increased diaphyseal BMD		[15]
Daidzein	10mg/kg body weight/day	Diet	OVX rat Wistar 12mo	90 days	No	0.23% of diet			Increased LS, total femur, diaphyseal & metaphyseal BMD		[15]

Phyto source	Dose	Mode	Model	Study length	Soy protein present?	Dietary calcium content	Outcome				Ref
							Calcium Balance	Biochemical Markers	BMC/ BMD	Other	
Growing sham-operated rat											
Genistein	5µg/g body weight (5mg/kg body weight)	s.c.	Sham rat Sprague Dawley 60 days (2mo)	21 days	No	0.6% of diet		No effect DPYD Increased serum osteocalcin	Increased tibial BMD.		[5]
Growing intact rat											
Isoflavones	0.046% of diet	Diet	Intact growing female rats Sprague Dawley 3wo	60 days	Yes	0.5% of diet	No effect calcium balance	No effect ALP activity, PYD or DPYDs			[16]
Isoflavones	0.046% of diet	Diet	Intact growing female rats Sprague Dawley 3wo	60 days	No	0.5% of diet	No effect calcium balance	No effect ALP activity, PYD or DPYDs			[16]
Genistein	5ppm	Diet	Intact male & female growing rats Sprague Dawley (4 generations)	2 years	No	1.15% of diet			Reduced caudal vertebrae BMC & BA		[17]

Phyto source	Dose	Mode	Model	Study length	Soy protein present?	Dietary calcium content	Outcome				Ref
							Calcium Balance	Biochemical Markers	BMC/ BMD	Other	
Genistein	100ppm	Diet	Intact male & female growing rats Sprague Dawley (4 generations)	2 years	No	1.15%		Increased urinary PYD excretion.	Reduced caudal vertebrae BMC & BA		[17]
Genistein	500ppm	Diet	Intact male & female growing rats Sprague Dawley (4 generations)	2 years	No	1.15%	.	Increased urinary PYD excretion	Reduced LS & caudal vertebrae BMC & BA Reduced cross-sectional area of femur		[17]
Isoflavone extract	8.9mg aglycones/kg body weight/day	Diet	Intact female F344 rat 3mo	14 weeks	No	0.85% of diet		No effect DPYD	No effect femur BMD		[18]
Isoflavone extract	18.1mg aglycones/kg body weight/day	Diet	Intact female F344 rat 3mo	14 weeks	No	0.85% of diet		No effect DPYD	Slight increase LS BMD No effect femur BMD	,	[18]
Soy protein	10.1mg aglycones/kg body weight/day	Diet	Intact female F344 rat 3mo	14 weeks	Yes	0.85% of diet		No effect DPYD	Increased LS BMD No effect femur BMD		[18]

Phyto source	Dose	Mode	Model	Study length	Soy protein present?	Dietary calcium content	Outcome				Ref
							Calcium Balance	Biochemical Markers	BMC/ BMD	Other	
Soy protein	20.2mg aglycones/kg body weight/day	Diet	Intact female F344 rat 3mo	14 weeks	Yes	0.85% of diet		Decreased urinary DPYD.	No effect femur BMD Slight increase LS BMD.	Increased uterine weight	[18]
Aged sham-operated rat											
Isolated soy protein	140g/kg diet isolated soy protein Isoflavone content not reported	Diet	Sham rat Sprague Dawley 11mo (retired breeders)	3 months	Yes	Not reported			No effect femoral BMD		[14]
Non-rodent OVX											
Soy protein isolate	20g soy protein/100g diet	Diet	OVX macaques (age unknown)	7 months	Yes	Not reported				No effect periosteal, osteonal or total bone formation rates Increased endocortical bone turnover	[19]
Soy protein isolate with isoflavones	35-40mg isoflavones/monkey/day Equivalent to 10-11mg/kg body weight/day	Diet	OVX monkeys	3 years	Yes	830mg/day		Slight reduction in serum CTX & ALP at 3 months but not at other timepoints	No effect whole body or LS BMC or BMD		[20]

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Appendix 3:

The ovariectomised rat model for post-menopausal bone loss

The ovariectomised rat is a widely used model for postmenopausal bone loss. Various studies have reported similarities between ovariectomy-induced bone loss in rats and postmenopausal bone loss in humans [1, 2]. Shared characteristics include:

- increased bone turnover rate with rate of resorption exceeding that of formation [2, 3]
- initial rapid bone loss followed by a much slower rate of bone loss
- greater loss of trabecular compared to cortical bone [4]
- decreased intestinal calcium absorption [3]
- protective effect of obesity [3]
- similar responses to treatment with oestrogen, bisphosphonates, parathyroid hormone, calcitonin and tamoxifen [3]
- protective effect of exercise [3].

Bone loss as a result of ovariectomy in rats or menopause in women occurs in two phases. Phase 1 is an acute phase characterized by a rapid decline in bone mineral content [5, 6]. Bone loss continues in phase 2 but occurs at a much slower rate than in phase 1. The aetiology of bone loss in phase 1 differs from that in phase 2 [6-8].

In both humans and rats the acute phase of bone loss is characterized by increased urinary calcium excretion [6, 7]. In rats this phase lasts for approximately 4-6 weeks post-ovariectomy [7] whereas is women, this rapid phase of bone loss continues for approximately 5-10 years from the initial onset of natural menopause or following surgical menopause [3, 9]. By week six post-ovariectomy in rats, faecal calcium loss appears to be of much greater importance than urinary loss in terms of its association with the decline in bone calcium [7, 8]. However there is no evidence of impaired intestinal calcium absorption until nine weeks post-ovariectomy [7] indicating the initial increase in faecal and urinary calcium excretion is a result of endogenous calcium loss. In women, a similar increase in bone turnover leading to endogenous calcium loss occurs in the acute phase post-menopause. Reduced intestinal calcium

absorption also occurs in women and is believed to contribute to post-menopausal bone loss [9].

Limitations of the ovariectomised rat model

At the endocrine level, there are key differences between the pathogenesis of postmenopausal bone loss in women and ovariectomy-induced bone loss in rats. In women, decreased parathyroid hormone (PTH) secretion, increased calcitonin secretion and impaired 1,25-dihydroxyvitamin D3 synthesis contribute to post-menopausal bone loss [9]. In rats, a negative correlation between calcitonin, but not PTH, secretion and bone resorption post-ovariectomy has been reported [10]. Synthesis of 1,25-dihydroxyvitamin D3 is unaltered in rats post-ovariectomy however expression of the vitamin D receptor is reduced which leads to disrupted vitamin D signaling post-ovariectomy [11]. Both ovariectomy in rats and menopause in women are associated with an increase in inflammatory cytokine secretion [9, 12] and inflammation is believed to play a major role in the aetiology of bone loss [12].

Long bones in rats continue to grow well past the attainment of maturity. In female rats, long bone growth ceases at approximately 6 months of age [3, 13]. In comparison to humans, the skeletal mass of a rat remains constant for a longer period of time therefore rats are susceptible to age-related changes in bone metabolism at a later stage of their life cycle than humans [3]. As a consequence, the ovariectomised rat principally exhibits the effects of oestrogen deficiency whereas postmenopausal women are likely to exhibit the effects of oestrogen deficiency as well as age-related metabolic changes. In addition, bone loss post-ovariectomy in rats seldom leads to increased fracture risk whereas an increase in fracture risk is one of the primary features of post-menopausal osteoporosis [3]. There is evidence that ovariectomy-induced changes in bone metabolic rates in rats mimic those of postmenopausal trabecular bone for a relatively short period of time. By 12 months post-ovariectomy indices of trabecular bone turnover in rats return to those of sham controls [4, 14] and bone strength reverts to that of sham animals at age 1 year [4].

The impact of these differences in bone metabolism and bone loss following ovariectomy in rats and menopause in women can be minimized by the use of rats with a minimum age of 6 months at the time of ovariectomy and by limiting the study to < 1year in duration.

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Appendix 4:

The MC3T3-E1/4 cell line as a model for osteoblasts *in vivo*

Prior to establishment of the MC3T3-E1 cell line, only one mammalian cell system capable of mineral deposition existed [1].

In 1981, Yamamoto *et. al.* established eight clonal cell lines from newborn mouse (Mus musculis) calvaria [1]. Based on its high alkaline phosphatase activity in the resting state, the cell line named MC3T3-E1 was identified as having the greatest osteogenic capability [1].

MC3T3-E1 cells have typical fibroblast morphology whilst in the logarithmic growth phase. During proliferation, cells express low alkaline phosphatase activity and do not produce an extracellular collagen matrix [2]. Once confluent, cells become cuboidal in shape and begin to synthesise and exude collagen and deposit mineral [1, 2]. There is considerable heterogeneity in terms of cell phenotype [3] and mineralisation potential [4] within the MC3T3-E1 cell line.

In 1999, researchers at the University of Michigan sub-cloned the MC3T3-E1 cell line and separated sub-clones based on their ability to differentiate into mature osteoblasts capable of matrix mineralisation [3]. Sub-clones 4 and 14 were determined to have the greatest differentiation and mineralisation potential [3]. Since this time, the MC3T3-E1/4 cell line has been utilised extensively for assessing the activity of osteoblasts *in vivo.*

MC3T3-E1/4 cells exhibit many of the characteristics and capabilities of osteoblasts. In vitro, MC3T3-E1/4 cells will proliferate when grown in minimum essential media in the presence of 5-10% FCS. Ascorbic acid is required for production of a collagenous extracellular matrix and β-glycerophosphate is needed for mineralisation of this matrix [4]. The role of β-glycerophosphate in mineralisation is not fully understood. It is a substrate for alkaline phosphatase however and there is some suggestion that β-glycerophosphate alters the phosphorylation state of casein kinase II, one of the enzymes thought to be involved in mineralisation. However even with

the use of the sub-clone, considerable heterogeneity exists in cell phenotype and this heterogeneity increases with repeated passage [3]. This is a limitation which must be considered when using MC3T3-E1/4 cells. In the present thesis, studies were conducted using MC3T3-E1/4 cells between passage 2 and 15.

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List of Publications

Peer-Reviewed Scientific Papers

Poulsen RC, Moughan PJ, Kruger MC (2007) Mini-Review: Long chain polyunsaturated fatty acids and the regulation of bone metabolism. **Experimental Biology and Medicine** (accepted)

Poulsen RC, Firth EC, Rogers CW, Moughan PJ, Kruger MC (2007) Specific effects of gamma-linolenic, eicosapentaenoic and docosahexaenoic ethyl esters on bone post-ovariectomy in rats. **Calcified Tissue International** (accepted)

Poulsen RC, Wolber FM, Moughan PJ, Kruger MC (2007) Long chain polyunsaturated fatty acids alter membrane-bound RANK-L expression and osteoprotegerin secretion by MC3T3-E1 osteoblast-like cells. **Prostaglandins and other Lipid Mediators** (accepted)

Poulsen RC, Moughan PJ, Kruger MC (2007) Docosahexaenoic acid and 17β-estradiol co-treatment is more effective than 17β-estradiol alone in maintaining bone post-ovariectomy **Experimental Biology and Medicine** (submitted)

Poulsen RC, Gotlinger KH, Serhan CN, Kruger MC (2007) Identification of inflammatory and pro-resolving lipid mediators in bone marrow and their profile alteration with ovariectomy and omega-3 intake **American Journal of Hematology** (submitted)

Conference Presentations

Poulsen RC, Firth EC, Rogers CW, Kruger MC (2006) Specific effects of gamma-linolenic, eicosapentaenoic and docosahexaenoic ethyl esters on bone post-ovariectomy in rats**. ISSFAL (International Society for the Study of Fatty Acids and Lipids)**, Cairns, Australia

Poulsen RC, Firth EC, Rogers CW, Moughan PJ, Kruger MC (2006) Specific effects of gamma-linolenic, eicosapentaenoic and docosahexaenoic ethyl esters on bone post-ovariectomy in rats. **ANZBMS (Australia New Zealand Bone and Mineral Society)**, Port Douglas, Australia

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