An Investigation into the Effects of Omega-3 Fatty Acids on Bone Resorption in the Female Ovariectomised Rat

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Abstract

Estrogen deficiency results in disruption of the normal bone remodeling cycle leading to a loss of bone mineral and, in many cases, the development of osteoporosis. Various studies have demonstrated a beneficial effect of essential fatty acids (EFAs) in reducing the loss of bone density as a consequence of estrogen deficiency. The aim of the present study was to examine the specific effects of the n-3 EFA, eicosopentaenoic acid (EPA) on bone density and strength in ovariectomised female rats.

60 Sprague-Dawley rats were randomized into four groups and either ovariectomised (n=45) or sham operated (n=15). Ovariectomised animals were fed calcium adequate diets containing either corn oil (OVX control, n=15), corn oil + 0.1g/kg body weight EPA (low dose, n=15) or corn oil + 1.0g/kg body weight EPA (high dose, n=15) for a period of nine weeks. Sham rats were fed the corn oil diet as per the OVX control group. Urinary calcium and phosphate excretion, serum type 1 collagen c-telopeptide concentration, bone density, bone ash and bone breaking strength were measured. Plasma fatty acid composition and serum concentrations of 25 hydroxyvitamin D_3 were also determined.

Femur bone density was significantly lower in the high dose group compared to sham, OVX control and low dose EPA groups ($p<0.001$, $p=0.0096$ and $p=0.0047$ respectively). Low dose EPA supplementation had no significant effect on bone density. No significant differences in urinary calcium or phosphate concentrations, serum concentrations of type-1 collagen c-telopeptide or bone breaking strength were evident with either dose of EPA compared to unsupplemented, ovariectomised controls. EPA supplementation resulted in significant decreases in the levels of n-6 EFAs and increases in the levels of n-3 EFAs except docosahexaenoic acid in plasma lipids. Both low and high dose EPA supplementation led to significant increases in serum concentration of 25(OH) vitamin D_3.

In conclusion 1.0g EPA/kg body weight had a detrimental effect on bone density in ovariectomised rats. It is proposed that high intake of the highly unsaturated EPA resulted in significant lipid peroxidation. This in turn disrupted membrane structure and inhibited

intestinal calcium absorption thereby stimulating PTH-mediated bone resorption. A potential role for n-3 EFAs in the regulation of vitamin D activity is also outlined.

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List of Abbreviations

$1,25(OH)_2D_3$	1,25 dihydroxyvitamin D_3
$25(OH)vitD_3$	25 hydroxyvitamin D_3
AA	Arachidonic Acid (20:4n-6)
ALA	Alpha-Linolenic Acid (18:3n-3)
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphatase
BGP	Bone Gla Protein (osteocalcin)
BMI	Body Mass Index
BMP	Bone Morphogenic Protein
BRU	Bone Remodelling Unit
Ca or Ca^{2-}	calcium
cAMP	Cyclic Adenosine Monophosphate
Cbfa-1	Core Binding Factor 1
Cl	Chloride
CLA	Conjugated Linoleic Acid
COX	Cyclooxygenase
CTX	C-terminal telopeptide of type-1 collagen
DHA	Docosahexaenoic Acid (22:6n-3)
DLX-5	Distal-less 5 transcription factor
DPA	Docosapentaenoic Acid (22:5n-3)
Dpyd	Deoxypyridinoline
EFA	Essential Fatty Acid
EGF	Erythrocyte Growth Factor
ELISA	Enzyme-linked Immunoassay
EPA	Eicosapentaenoic Acid (20:5n-3)
FGF	Fibroblast Growth Factor
g	gram
GLA	Gamma Linolenic Acid (18:3n-6)

gp130	Glycoprotein 130
GTPase	Guanisine Triphosphatase
H^-	Hydrogen
hGH	Human Growth Hormone
HMG-CoA	Hydroxymethylglutaryl Coenzyme A
IFN	Interferon
IGF	Insulin-like Growth Factor
IGFBP	Insulin-like Growth Factor Binding Protein
IL	Interleukin
IV	intravenous
K or K^+	Potassium
kg	kilogram
LA	Linoleic Acid (18:2n-6)
LT	Leukotriene
LTB4	Leukotriene B4
LTB5	Leukotriene B5
M-CSF	Monocyte-Macrophage Colony Stimulating Factor
mg	milligram
Mg or Mg^{2+}	Magnesium
mL	milliliter
mm	millimeter
mMol	millimoles
MMPs	Matrix Metalloproteinases
N	Newton
n-3	omega 3
n-6	omega 6
n-9	omega 9
Na or Na^+	Sodium
NF-κB	Nuclear Factor-κB
ng	nanogram
N/mm^2	Newtons per square millimeter

OPG	Osteoprotegerin
OVX	ovariectomised
PDGF	Platelet-derived Growth factor
PGE_2	Prostaglandin E2
PGE_3	Prostaglandin E3
PKC	Protein Kinase C
PO_4	Phosphate
POV	Peroxide Value
PPAR	Peroxisome Proliferator Activated Receptor
PPRE	Peroxisome Proliferator Response Element
PTH	Parathyroid Hormone
PTHrp	Parathyroid Hormone-related protein
PUFA	Polyunsaturated Fatty Acid
RANK-L	RANK ligand
RXR	Retinoid X Receptor
SD	Standard Deviation
SE	Standard Error
T3	Triiodothyronine 3
T4	Thyroxine
TGF	Transforming Growth Factor
TNF	Tumour Necrosis Factor
TRAFs	Tumour Necrosis Factor Receptor-Associated Factors
TxB2	Thromboxane B2
VDR	Vitamin D Receptor
WHO	World Health Organisation
Yrs	Years

Introduction

Darwin's theory of evolution assumes the purpose of an animal is to reproduce in order to ensure the survival of the species. Whether due to disease, predation, the trauma of childbirth or natural causes, it is rare for members of the animal kingdom to survive beyond the age at which they lose the ability to reproduce. It follows therefore that mechanisms within the body act to ensure health and survival for a finite period of time, namely until the animal is no longer reproductively active. Humans, however are unique in that they have adapted their environment in such a way as to virtually eliminate many of the causes of death which plague other members of the animal kingdom. Sanitation, an abundant food supply, modern housing and medical care have all worked to prolong human longevity by such an extent that the average lifespan of a women living in a developed country is nearly double that of her reproductive lifespan. Natural evolution results in selection of traits that promote the health and survival of an animal throughout its infancy and reproductive years. However, traits that specifically promote longevity passed the reproductive years will not be specifically past on to future generations.

As well as providing energy for the body, food is also a source of bioactive molecules and their precursors. These factors have an active role in regulating metabolic processes within the body therefore dietary choices are important determinants of overall health. In a healthy animal, hormones direct the activity of diet-derived 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 result in loss of this buffering action and diet, for good or ill, becomes an even more important determinant of overall health and well-being.

Loss of bone mineral was once considered an inevitable consequence of ageing. Particularly in post-menopausal women, bone mineral loss can result in the development of debilitating conditions such as osteoporosis. Furthermore, reduced bone mass may indicate an overall disturbance in the systems involved in determining the site of calcium deposition within the body. As a result, the calcification of soft tissue at the expense of

bone tissue may occur leading to arterial damage. A decrease of 1 SD in bone mineral density is associated with a 20% increase in all-cause mortality. The majority of the increased mortality is attributed to vascular disease (Kruger & Horrobin, 1997). Essential fatty acids are bioactive compounds which appear to be involved in the channeling of calcium in the body (Schlemmer,1997). Manipulation of the levels of essential fatty acids in the diet may be a means of combating the disruption in calcium regulation which occurs post-menopause thereby ensuring that calcium is deposited in bone rather than soft tissue.

CHAPTER 1

LITERATURE REVIEW

SECTION 1

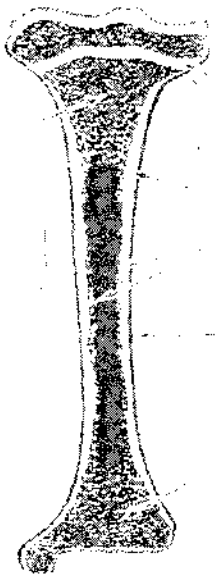
Bone Metabolism

1.1 Bone Structure and Function

When considered at all, the skeleton is generally thought of as simply an inert framework for the body - the "building" that houses the equipment which performs all the real work. It is in fact a dynamic structure constantly renewed throughout the lifecycle. Over a period of ten years the skeleton is completely broken down and rebuilt allowing it to continually adapt to the changing stresses placed on it throughout the lifetime. The skeleton is not just a framework for the body. It provides protection for the body's organs and also serves as a site for muscle attachment thereby enabling the body to move. Perhaps least known of all the skeleton's functions however is its role as a reservoir for ions - particularly calcium and phosphate. By serving as an ion store, the skeleton performs a crucial role in the maintenance of serum ion homeostasis and consequently in the regulation of metabolism.

There are two types of bone - cortical and trabecular. Cortical bone is the most abundant making up approximately 75-85% of total body bone (Baron, 1995). Cortical bone forms a dense outer shell that surrounds long bones. It is mainly responsible for the structural and mechanical functions of the skeleton. Within the shell created by cortical bone is a calcified web-like network referred to as cancellous or trabecular bone. The lattice-like structure of trabecular bone results in it having a large surface area. Hematopoietic bone marrow is found within the spaces of the trabecular bone lattice and 70-85% of the interface of bone with soft tissue occurs within trabecular bone. Trabecular bone is largely responsible for the regulation of serum ion concentration.

Fig 1 Cortical and Trabecular Bone



(Reproduced from Netter, 1987)

Different bones in the body are made up of different proportions of cortical and trabecular bone.

Fig 2 Proportions of cortical and trabecular bone in various parts of the skeleton

(Reproduced from Baron, 1995).

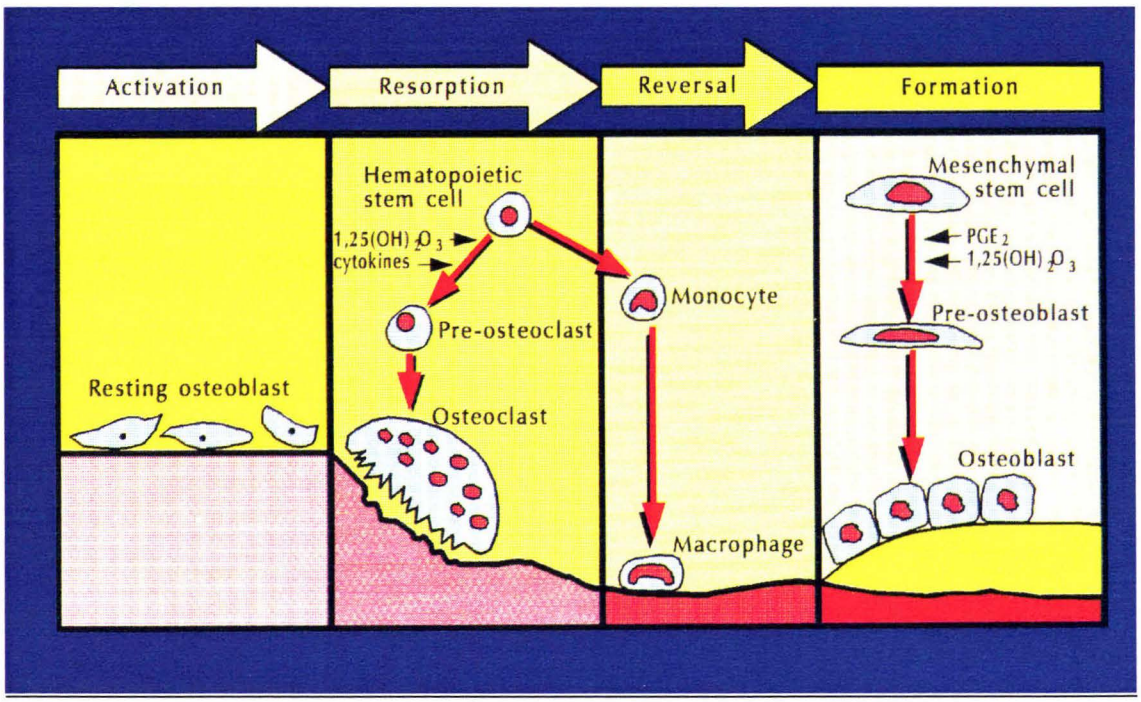
Approximately 30% of bone is organic tissue which has been secreted by specialized bone cells and organized in such a way as to form a matrix on which minerals can be deposited. This matrix predominately consists of type-I collagen fibres which provide tensile strength to the bone. Non-collagenous proteins such as osteocalcin (which binds calcium) constitute <10% of total bone protein (Baron, 1995). Crystals mainly comprised of calcium and phosphate form on the matrix providing the characteristic hardness of bone. The most stable and therefore most abundant crystal formed is hydroxyapatite ($3Ca_3(PO_4)_2.(OH)_2$). Cortical and trabecular bone are made up of the same cells and matrix elements but 80-90% of cortical bone is calcified compared to only 15-25% of trabecular bone (Baron, 1995).

Two metabolic processes operate at separate stages of the life cycle to govern bone growth and maintenance. Bone modeling is the process by which bones grow during childhood and adolescence and results in net accrual of bone tissue. Bone remodeling is the process by which bone is continuously turned over in order to maintain bone mass and integrity and to regulate body calcium levels (Watkins *et al*, 1996). Both processes involve resorption of existing bone tissue as well as formation of new bone. Bone homeostasis is maintained by regulation of the rates of genesis and apoptosis of bone forming (osteoblast) and bone-resorbing (osteoclast) cells (Manolagus, 2000). In bone modeling, bone resorption and formation occur independently of one another with rate of formation exceeding that of resorption. During bone remodeling the processes of resorption and formation are coupled with the net effect being no change in the total amount of bone mass (Watkins *et al*, 1996). In humans, an estimated 10% of bone is remodeled each year (Theill *et al*, 2002), the vast majority of which is trabecular bone (Manolagus *et al*, 1995). Mis-regulation of bone remodeling therefore can have important consequences for the maintenance of body ion homeostasis. Calcium imbalance is believed to be a key contributing factor for several pathological conditions such as osteoporosis and vascular disease (Das, 2000).

1.2 The Bone Remodeling Cycle

Understanding the Bone Remodeling Cycle is crucial to understanding the pathogenesis of age-related bone loss and osteoporosis. Bone remodeling occurs in discrete areas of the bone known as Bone Remodeling Units (BRU). Each BRU is geographically isolated from other BRUs suggesting that the site of remodeling is under the control of localized regulatory factors. Remodeling of each unit takes approximately 3-4 months. It commences with a period of osteoclastic-mediated bone resorption lasting 2-3 weeks. Formation of new bone by osteoblasts is a slower process and takes 2-3 months to complete (Baron, 1995).

Fig 3 The Bone Remodeling Cycle



(Reproduced from van Papendorp, 1993)

As shown in Figure 3, the resorption phase of the bone turnover cycle begins with the differentiation of osteoclasts from hematopoietic monocyte and macrophage precursors. Osteoclast precursors reach bone via the circulatory system (Manolagus, 2000) in response to chemical and/or electrical stimuli (Watkins *et al,* 1996).

1.2.1 Osteoclast Differentiation

Osteoclast differentiation appears to be controlled by the relative ratios of three proteins:

1. RANK-L, a trimeric protein expressed in high amounts by osteoblasts which exists in both a transmembrane and a soluble, free form
2. RANK, the membrane receptor for RANK-L which is expressed by hematopoietic osteoclast progenitors, mature osteoclasts, chondrocytes and mammary gland epithelial cells
3. Osteoprotegerin (OPG), a natural, soluble decoy receptor for RANK-L produced by osteoblasts as well as by follicular dendritic cells.

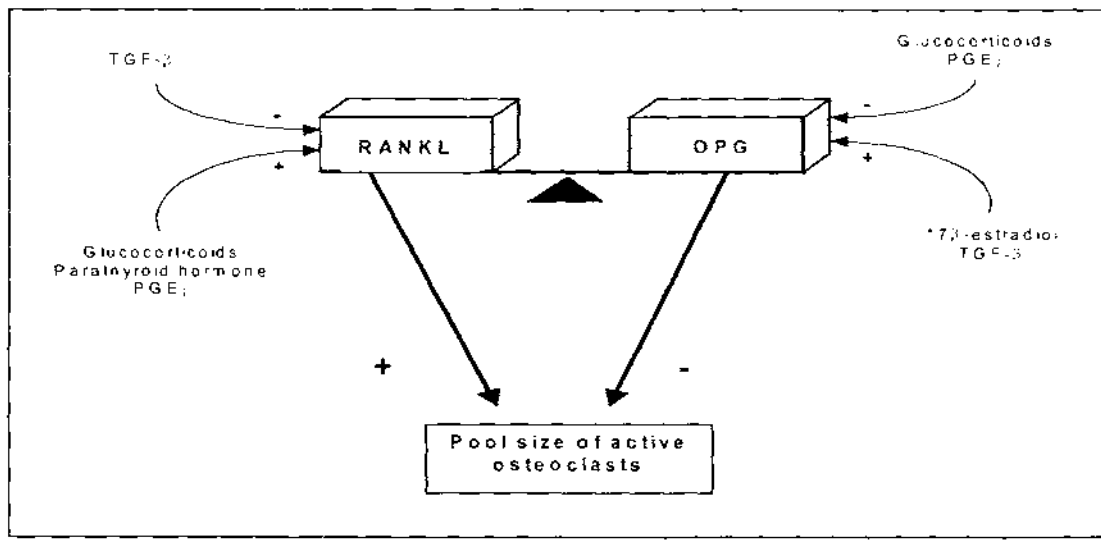
Binding of RANK-L to RANK on hematopoietic progenitors in the presence of Monocyte-Macrophage Colony Stimulating Factor (M-CSF) induces osteoclast differentiation via a signal transduction cascade involving specific signaling molecules known as Tumour Necrosis Factor Receptor-Associated Factors (TRAFs). c-Src and Cbl are proteins associated with the cytoplasmic tail of RANK which relay stimulatory signals to downstream second messenger pathways such as Nuclear Factor-κB (NF-κB) and JNK/SAPK. These in turn regulate bone resorption, and the activation, survival and differentiation of osteoclasts and dendritic cells. Binding of RANK-L to RANK also stimulates the activity of mature osteoclasts whereas binding of RANK-L to OPG inhibits osteoclastogenesis and activation of mature osteoclasts (Theill *et al,* 2002). The relative ratio of OPG and RANK-L is therefore important in determining the pool size of active osteoclasts as they compete with each other for RANK binding. This is known as the Convergence Theory and is depicted in Figure 4.

RANK-L is expressed in many different tissues eg lymph nodes, spleen thymus and intestinal lymphoid patches however osteoclast development only occurs in bone (Theill *et al,* 2002). This suggests that other factors are also involved in stimulating osteoclastogenesis in bone tissue. RANK-L and RANK are also expressed on chondrocytes and may have a role in cartilage growth and homeostasis (Theill *et al,* 2002).

1.2.1.1 Regulation of Osteoclast Differentiation

Both systemic hormones such as parathyroid hormone (PTH) and localized factors such as Transforming Growth Factor (TGF), and prostaglandins (particularly prostaglandin E2 (PGE_2)) control osteoclastic differentiation by regulating the relative ratio of RANK-L and OPG. Figure 4 illustrates the effects of some of these regulatory factors on osteoclastogenesis.

Fig 4 Control of Differentiation and Activation of Osteoclasts



(Reproduced from Hauffman et al. 2000)

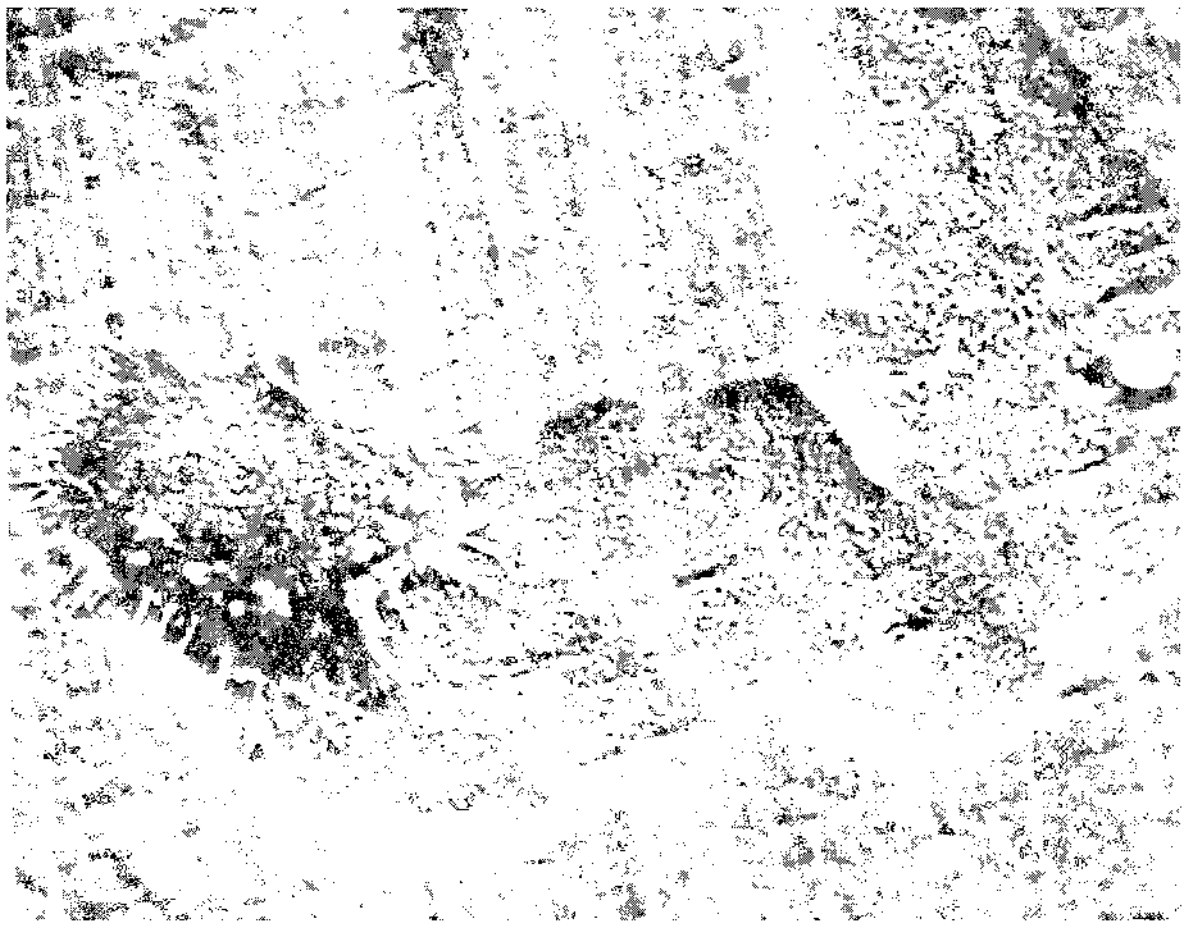
1.2.2 Osteoclast Activity

The second phase of bone resorption involves attachment of the newly-formed osteoclasts to the remodeling site. Integrins (integral membrane proteins) are proteins found on the osteoclast membrane which interact with Matrix Metalloproteins (MMPs) (a family of proteins present in the organic bone matrix) thereby affixing the osteoclast to the bone surface (Delaisse *et al.* 2000; Baron, 1995). A photo of an osteoclast attached to bone surface as viewed by an electron microscope is shown in Figure 5.

Mature osteoclasts are multinucleated cells which have a dense belt of actin surrounded by an enlarged area of plasma membrane known as the ruffled border membrane (Stenbeck, 2002). The ruffled border membrane creates a sealed microenvironment on a small area of

the bone surface. The membrane contains vacuolar H^+-ATPase which acidifies the sealed area and dissolves the inorganic constituents of bone (Katagiri, & Takahashi, 2002). Osteoclasts also secrete acidic proteinases which degrade the organic matrix (Blair *et al*, 2002) thereby resulting in formation of a cavity or lacuna in the bone.

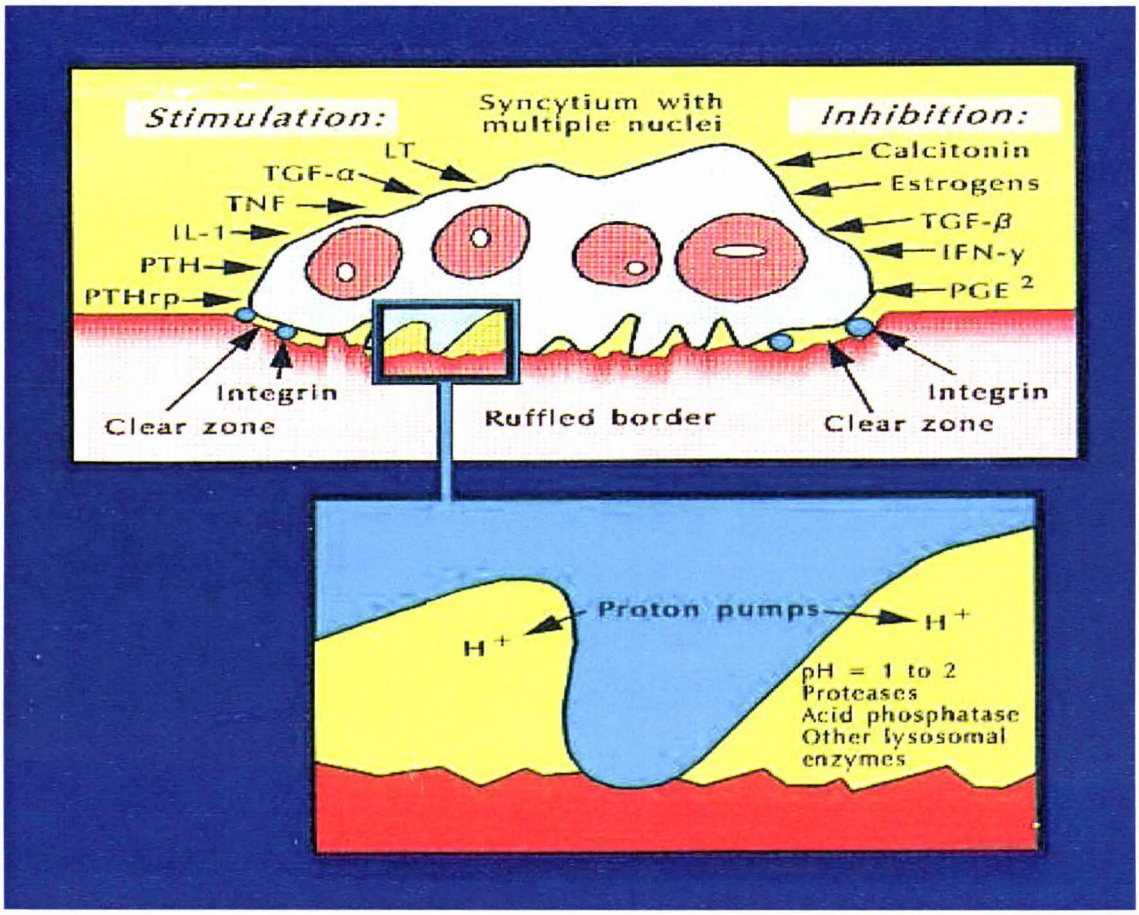
Fig 5 Electron Micrograph of an Osteoclast attached to Bone.



(Reproduced from Mundy, 1995)

Osteoclast activity is also under the control of both systemic and localized factors. As shown in Figure 6 estrogen inhibits and PTH promotes osteoclast activity. Cytokines such as interleukins, eicosanoids such as the prostaglandins and members of the Transforming Growth Factor superfamily all have a role in controlling osteoclast activation (Mundy, 1995).

Fig 6 Factors influencing Osteoclast Activation



(Reproduced from van Papendorp, 1993)

Once the resorption phase is complete, osteoclasts detach from the bone surface (reversal phase) allowing osteoblast-mediated bone formation to take place.

1.2.3 Osteoblast Differentiation

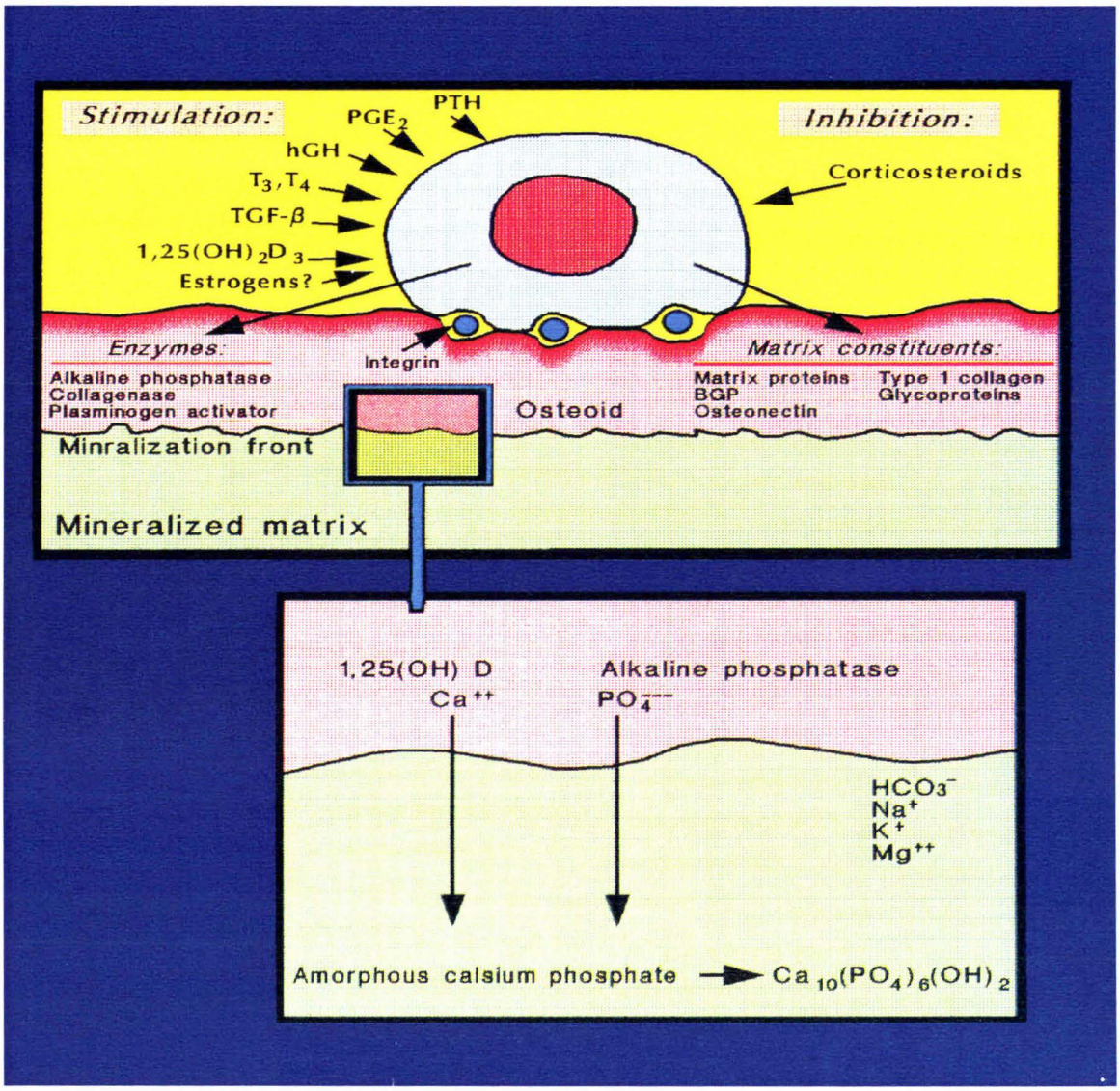
Osteoblast precursors migrate to bone from neighbouring connective tissues by following a concentration gradient of specific molecules such as the protein Sonic Hedgehog (Manolagus, 2000). Region-specific patterns of expression of a particular gene known as *hox* define the areas of initial precursor cell accumulation (Franceschi, 1999). Osteoblasts, along with adipocytes, myocytes and chondrocytes are derived from mesenchymal cells (Skillington *et al,* 2002). The mechanism which triggers mesenchymal cells to differentiate into osteoblasts as opposed to myocytes or adipocytes is as yet unknown. However it is

thought that mechanical stimulation may promote osteoblast and chondrocyte differentiation (Tang *et al,* 2002; Meyer *et al,* 2001). *hox* gene expression triggers mature osteoblasts to synthesise growth factors, prostaglandins and cytokines (Watkins *et al,* 2001) and deposit these in the extracellular bone matrix (Franceschi, 1999). During osteoclastic bone resorption these factors are released and stimulate differentiation of new osteoblasts (Katagiri & Takahashi, 2002). Bone Morphogenic Proteins (BMPs) are an example of such factors. BMPs are members of the Transforming Growth Factor super-family (Theill *et al,* 2002). BMP-RIA, BMP-RIB and BMP-2 acting in concert with retinoic acid promote proliferation and early differentiation of osteoblasts (Skillington *et al,* 2002). BMPs activate various transcription factors such as distal-less 5 (DLX-5) and Core Binding Factor-1 (Cbfa-1) thereby indirectly regulating bone mineralisation as well as the expression of osteoblast-specific genes eg osteocalcin and alkaline phosphatase (Katagiri & Takahashi, 2002; Manolagus, 2002).

1.2.3.1 Regulation of Osteoblast Differentiation

Growth Factors such as Transforming Growth Factor α (TGFα), Fibroblast Growth Factor (FGF) and Insulin-like Growth Factor (IGF) influence replication and differentation of committed osteoblast progenitors (Manolagus, 2000). The mechanism may involve regulation of N-cadherin-mediated cell-cell adhesion which is important in promoting osteoblast (as opposed to osteoclast) adhesion to bone matrix and controlling osteoblast apoptosis (Marie, 2002). As is the case with osteoclasts, osteoblast differentiation and activation is under the control of both systemic and localized factors. Many of the factors influencing osteoclast activity also regulate osteoblast activity eg PTH, estrogen, prostaglandins and transforming growth factors. Figure 7 illustrates the effects of various such regulators.

Fig 7 Factors influencing Osteoblast Differentiation and Activation



(Reproduced from van Papendorp, 1993)

1.2.4 Osteoblast Activity

Osteoblasts have two major roles in bone metabolism. Firstly they secrete factors into the extracellular matrix which are released during bone resorption and serve as autocrine and paracrine regulators of the bone turnover cycle. Osteoblasts also synthesise, secrete and organise components required for the formation of the organic bone matrix as well as for the mineralisation of bone tissue and the subsequent filling of lacunae (Watkins & Seifert, 1996).

1.2.5 Other Bone Cells

Once bone formation in a BRU is complete, there are two possible fates for the osteoblasts:

- To undergo apoptosis, i.e. programmed cell death.
- differentiation into one of two other types of specialized bone cells known as osteocytes and bone lining cells.

Osteocytes are believed to act as a network of mechanosensors providing feedback to the osteoblast to initiate bone modeling or remodeling (Gordeladze *et al,* 2001) possibly via gap junction communication (Watkins *et al,* 2001). The function of bone-lining cells is unknown but they may have a role in activation of bone re-modeling and are possibly capable of differentiating again into viable osteoblasts (Watkins *et al,* 2001). Bone-lining cells also appear to have a key function in the maintenance of serum calcium homeostasis as a result of their role in forming the osteocytic membrane.

1.2.5.1 The Osteocytic Membrane

Canicular channels exist within bone. These channels are the remnants of the vascular system that supported osteoblast activity during the bone formation phase. A special type of fluid simply referred to as "bone fluid" circulates within the canicular system and bathes the bone surface. The osteocytic membrane separates the bone fluid from the extracellular fluid (Puzas, 1995). Approximately 1% of total body calcium is contained in bone fluid. This is known as the "exchangeable calcium pool" as it is the only portion of skeletal calcium which is freely exchangeable with the extracellular fluid (Broadus, 1995). The osteocytic membrane acts as a buffer against short-term fluctuations in extracellular calcium concentration (Mundy, 1995). Aside from its role in maintaining serum calcium balance, there is some evidence that osteocyte-mediated regulation of bone fluid flow through individual bone compartments is one of the means by which calcitonin and parathyroid hormone exert their effects on bone formation. Parathyroid hormone decreased the hydraulic conductivity of cultured cells whereas calcitonin exhibited a biphasic effect - low concentrations increased hydraulic conductivity whereas high concentrations decreased it (Hillsley & Frangos, 1996).

1.3 Regulation of Bone Metabolism

Various factors influence bone metabolism by impacting osteoblast or osteoclast activity and/or survival.

1.3.1 Lifestyle Factors

Exercise and nutrition are two of the most important lifestyle factors affecting bone metabolism. Load-bearing exercise stimulates bone mass accretion whereas high alcohol intake, smoking and malnutrition inhibit bone formation. Many different dietary factors such as vitamin C, protein/amino acid and phosphate intake influence bone metabolism to various extents (Brown *et al.* 2002). 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. The majority (99%) of total body calcium is stored in bone and the body's calcium status is an important trigger determining the synthesis and activity of various hormones, such as PTH, calcitonin and $1,25(OH)_2$ vitamin D_3 that are involved in regulating bone metabolism (Netter, 1987).

1.3.2 Hormonal Regulation

Regulation of bone homeostasis occurs due to the effects of systemic hormones as well as via the action of several localized compounds. Systemic regulators include insulin, growth hormone and estrogen which promote bone formation; thyroid hormone, and glucocorticoids which stimulate bone resorption; calcitonin which inhibits bone resorption and PTH and $1,25\text{-}(OH)_2$-vitamin D_3 which stimulate both bone formation and resorption (Watkins *et al.* 2001). The relative ratio of systemic factors governs whether bone modeling or remodeling occurs and the rate at which it occurs thereby providing homeorrhetic regulation of bone metabolism. Hormonal regulation is partially achieved by modulation of the ratios and activities of various localised factors of bone metabolism.

1.3.2.1 Estrogen and Growth Hormone

Growth Hormone appears to regulate bone metabolism both directly and indirectly. Bone cells have a limited number of growth hormone receptors allowing direct binding of the hormone to the cells. Growth hormone also stimulates the synthesis of Insulin-like Growth Factor (IGF-1), a polypeptide which promotes bone formation and inhibits its degradation (Canalis, 1993). Whilst growth hormone acts primarily to stimulate bone formation, estrogen promotes bone formation but also inhibits bone resorption. Estrogen slows the activation frequency of osteoclasts on bone resorbing surfaces. It may also decrease the sensitivity of bone cells to PTH (Mundy, 1995).

1.3.2.2 Insulin

Although primarily involved with glucose metabolism, insulin also influences bone metabolism. It stimulates osteoblastic collagen synthesis, reduces renal calcium and sodium reabsorption and decreases urinary phosphate excretion (Stipanuk, 2000). Insulin increases the activity of mature osteoblasts. It does not influence osteoblast number and appears to have no effect on osteoclast number or activity (Canalis, 1993). It is necessary for normal bone mineralisation and it also stimulates production of IGF-1 in the liver (Canalis, 1993).

1.3.2.3 Parathyroid Hormone and Vitamin D_3

The activities of PTH and 1,25$(OH)_2$ vitamin D_3 are closely interrelated. Both hormones act on the intestine, bone and kidney in order to increase serum calcium concentration and serum calcium concentration is the main regulator of PTH secretion. Low serum calcium levels promote PTH secretion which in turn increases renal calcium reabsorption and induces the release of calcium from the rapidly turned over exchangeable calcium pool in bone fluid. After several hours, PTH stimulates osteoclastic activity and calcium is released from the bone itself (Mundy, 1995). PTH activity is complex however as low, intermittent doses of PTH promote collagen synthesis and bone formation whereas high continuous release of PTH inhibits type-1 collagen formation, stimulates collagen breakdown and promotes bone resorption. The mechanism by which PTH acts is not fully

understood although it appears that cAMP plays a crucial role. PTH stimulates a rapid increase in cAMP levels and administration of cAMP analogues can elicit many of the same effects as administration of PTH (Kronenberg, 1993). The anabolic effect of PTH appears to be largely due to the ability of PTH to increase IGF-1 synthesis (Finkelstein, 1996) again as a result of the cAMP signaling pathway (Partridge & Winchester, 1996). PTH also utilizes at least one other cAMP-independent signaling pathway involving protein kinases. It is possible that different signaling pathways are utilized in different cells in response to intermittent versus continuous PTH secretion thereby resulting in the different physiological effects evident as a result of intermittent versus continuous exposure to PTH (Finkelstein, 1996).

PTH also stimulates the activity of 1α-hydroxlase thereby increasing renal synthesis of the most active form of vitamin D_3, 1,25$(OH)_2$ vitamin D_3, from its precursor 25(OH) vitamin D_3. Conversely, 1,25$(OH)_2$ vitamin D_3 inhibits PTH secretion. It also increases intestinal calcium absorption. There are three basic stages involved in the transport of calcium across the intestinal membrane – transport across the apical membrane, through the cytoplasm and finally across the basolateral membrane. In order to ensure calcium transport occurs uni-directionally, these stages have an increasing affinity for Ca^{2+} in a ratio of 1:4:10 respectively (Haag & Kruger, 2001). In the first stage, Ca^{2+} enters the brush border via channels in the membrane. The calcium ions then bind to calmodulin, a protein which is associated with myosin I. Myosin I is a Mg^{2+} ATPase mechanoenzyme which binds to actin in the microvillar membrane. Binding of actin to myosin closes the Ca^{2+} channel in the brush border membrane. The channel is re-opened again upon the transfer of Ca^{2+} to calbindin, a calcium-binding protein responsible for transfer of Ca^{2+} across the cytoplasm. Basolateral transport of Ca^{2+} occurs by three different pumps: a Ca^{2+} Mg^{2+} dependent ATPase, a Na^{+}, Ca^{2+} exchanger or by a Ca^{2+} ATPase. 1,25$(OH)_2$ vitamin D_3 promotes the synthesis of calbindin and stimulates the expression of Ca^{2+}, Mg^{2+}-dependent ATPase (Haag & Kruger, 2001). Arachidonic acid promotes the activation of Ca^{2+}-ATPase (Haag & Kruger, 2001) and there is some evidence that 1,25$(OH)_2$ vitamin D_3 selectively incorporates arachidonic acid into membranes (Baggio *et al*, 2000). It is likely therefore that 1,25$(OH)_2$ vitamin D_3 also indirectly upregulates Ca^{2+}-ATPase activity and

therefore influences all three stages of active calcium uptake. In the kidney, $1,25(OH)_2$ vitamin D_3 promotes urinary calcium excretion. In bone, $1,25(OH)_2$ vitamin D_3 promotes osteoblastogenesis and therefore stimulates bone formation (Norman & Henry, 1993). These latter two functions of $1,25(OH)_2$ vitamin D_3 possibly serve as "overflow" mechanisms which exist in order to ensure serum calcium levels do not rise above the tightly regulated physiological threshold before negative feedback mechanisms are activated.

1.3.3 Localized Regulation

Localized factors include cytokines, growth factors, eicosanoids and interleukins (Watkins *et al,* 2001). As bone resorption/formation occurs at various isolated sites concomitantly it appears that localized factors may be involved in determining the site of bone turnover. Of the localized factors prostaglandins appear to one of the primary means of regulating bone metabolism (Watkins *et al,* 2001). Trabecular bone, due to its close proximity to the circulatory system, appears to be more sensitive to the influence of local factors on bone metabolism whereas cortical bone turnover is regulated more by systemic factors (Baron, 1995). The roles of localised regulators of bone metabolism, particularly eicosanoids and cytokines, are discussed in more detail in Section 3.

Table 1 Localised Regulators of Bone Metabolism

EFFECT ON BONE	
Anabolic	**Catabolic**
Prostaglandins particularly PGE2 (low concs)	Interleukins (IL)
Growth Factors eg IGF-1, TGF	Prostaglandins particularly PGE2 (high concs)
Bone Morphogenic Proteins (BMPs)	Colony Stimulating Factor (CSF)
Interferon-γ	Tumour Necrosis Factor (TNF)

(Summarised from Watkins et al, 2001)

SUMMARY

Bone is continually remodeled throughout the lifespan in order to ensure ongoing functionality of the skeleton as both a support mechanism and an ion store. Remodeling occurs via a cyclical process mediated by osteoclasts and osteoblasts and commencing with the resorption of existing bone tissue. The aim of remodeling is to maintain existing bone mass. This is achieved by balancing the rate of bone formation with that of resorption through mediation of the synthesis and apoptosis of osteoclasts and osteoblasts. Hormones such as estrogen and PTH and localised regulators such as cytokines and prostaglandins are involved in regulating bone turnover.

A Bone Remodeling Cycle is initiated through the induction of osteoclastogenesis from precursor cells as a result of the action of an external force on bone. This may be in the form of a mechanical stimulus or a chemical messenger. Osteoclast differentiation is regulated by various systemic and localised factors which control the relative ratios of the two proteins RANK-L and OPG. Factors that promote RANK-L synthesis over that of OPG promote osteoclastogenesis and osteoclast activity. Many of the same factors that promote osteoclast differentiation and activity have the opposite effect on osteoblast differentiation and activity. Some of the most important agents regulating both osteoclasts and osteoblasts are estrogen, PGE-2 and TGF-β. Osteoclast activity releases growth factors and other regulatory agents from the bone matrix which were deposited by osteoblasts during the previous remodeling cycle. These factors act in an autocrine/paracrine fashion to initiate osteoblastogenesis and also regulate osteoclastic activity. To a certain extent therefore, bone remodeling occurs in response to signals stemming both from the current situation at the remodeling site (due to the initial external signal promoting osteoclast differentiation and attachment at the remodeling site) as well as signals resulting from the previous situation at the remodeling site (due to the release of regulators deposited in the matrix during the preceding remodeling cycle). As remodeling at any one site may occur as little as once every 10 years, a relatively sudden major change in the bone environment may disrupt this regulatory process. Trabecular bone, due to its higher rate of turnover may exhibit such an effect earlier than cortical bone. As trabecular bone is largely responsible for the maintenance of serum calcium levels and calcium is an important cell messenger

and regulator of many biological processes, mis-regulation of bone metabolism can have wide-reaching consequences on overall health.

SECTION 2

Mis-Regulation of Bone Metabolism

By far the most common condition resulting from mis-regulation of bone turnover in adults is osteoporosis.

1.4 Definition of Osteoporosis

The 1993 Consensus Development Conference provided the following definition of osteoporosis: "a disease characterized by low bone mass and microarchitectural deterioration of bone tissue leading to enhanced fragility and a consequent increase in fracture risk" (Stanley, 2001). The World Health Organization defines osteoporosis as a bone mineral density 2.5 SD below the mean peak value in young adults (Stanley, 2001). The risk of suffering an osteoporotic fracture over the course of a lifetime is estimated as being 40% for women and 13% for men. The most common fractures resulting from osteoporosis are of the hip and vertebrae (World Health Organization, 1999). WHO predict that the number of hip fractures worldwide as a consequence of osteoporosis will rise from 1.7million in 1990 to 6.3million by 2050 (World Health Organization, 1999*). Osteoporosis affects more than half of all New Zealand women and almost a third of New Zealand men over the age of 60. More than 3000 New Zealanders suffer from hip fracture each year (Osteoporosis New Zealand, 2003). Approximately one third of those who suffer a hip fracture die within the next year from related complications and another third remain hospitalized or institutionalized. Many of those who do return home have impaired mobility and as a result, a loss of independence (Osteoporosis New Zealand, 2003).

1.5 Pathogenesis of Osteoporosis

There are three main causes of mis-regulation leading to osteoporosis:

1. **Glucocorticoid excess** This is the least common of the three causes listed. It is a result either of a genetic disorder known as Cushing's Syndrome or of the use of

glucocorticoid drugs as are often prescribed for the treatment of arthritis (Stipanuk, 2000). The result is suppression of osteoblastogenesis in bone marrow due to concurrent inhibition of OPG and promotion of RANK-L expression, initial promotion of osteoclast survival followed by increased rates of apoptosis of osteoclasts, osteoblasts and osteocytes (Manolagus, 2000).

2. **Senescence (Senile Osteoporosis)** - Decreased osteoblastogenesis, increased adipogenesis and myelopoiesis and decreased osteoclastogenesis occur with advancing age. This may be a result of reduced levels of growth hormone and therefore IGF (Manolagus, 2000). It primarily results in loss of cortical bone (Manolagus & Jilka, 1995).
3. **Sex steroid deficiency** - this occurs in castrated males (androgen deficiency) and post-menopausal women (estrogen deficiency) (Manolagus, 2000). This form of osteoporosis is associated with formation of deeper than usual lacunae which are incompletely filled with new bone tissue by osteoblasts. It commonly affects trabecular rather than cortical bone (Baron, 1995).

Postmenopausal osteoporosis is the most common form of osteoporosis affecting 1 in 4 women (Stanley, 2001). Estrogen deficiency is believed to be responsible for an estimated 52.5-66.4% of the bone mineral loss occurring in the first 20 years of natural menopause (Gnudi *et al.* 1990).

There are two main theories as to the pathogenesis of post-menopausal osteoporosis:

1. Impaired coupling between bone resorption and formation
2. Decline in the amount of bone growth factors deposited in the bone matrix resulting in a decrease in rate of bone formation (Seifert & Watkins, 1997).

1.5.1 The Uncoupling Theory

According to the uncoupling theory, estrogen deficiency leads to an imbalance in levels of various localized regulatory factors which are responsible for coupling the processes of bone resorption and formation. The agent(s) responsible for the coupling process have yet

to be identified however many different candidates have been suggested all of which are regulated to some extent by estrogen.

Members of the Transforming Growth Factor (TGF) superfamily have been implicated as potential coupling agents. TGF-β is produced by osteoblasts and secreted into the extracellular bone matrix. It is released from the matrix during osteoclast-mediated degradation of bone tissue. TGF-β is a chemo-attractant therefore it aids in recruiting osteoblasts to the bone-remodeling site. It also stimulates proliferation of osteoblast precursors. Bone Morphogenic Protein-2 (BMP-2), another member of the TGF superfamily, is also produced by osteoblasts and released from the matrix during bone resorption. BMP-2 initiates differentation of precursor cells into mature osteoblasts (Mundy, 1995).

Other growth factors implicated as possible coupling agents include IGF-1, IGF-2, Platelet Derived Growth Factor (PDGF), and Fibroblast Growth Factor (FGF). All of which have been shown to stimulate proliferation of osteoblast precursors. PDGF also appears to have a role in chemotaxis (Mundy, 1995) .

Estrogen deficiency results in increased synthesis and release of localized regulatory factors such as inflammatory cytokines and decreased synthesis of osteoblast-derived growth factors such as those belonging to the TGF superfamily. The resultant imbalance in the levels of localized agents responsible for regulation of bone remodeling may lead to uncoupling of the rates of resorption vs formation. This may particularly impact trabecular bone due the greater relative influence of localized rather than systemic agents on the regulation of trabecular bone turnover (Manolagus & Jilka, 1995).

1.5.2 Decline in the Level of Growth Factors in Bone Matrix

This theory is based on the same observations as the uncoupling theory ie a decrease in the amount of osteoblast-derived growth factors deposited in the bone matrix results in a reduction in the rate of bone formation leading to osteoporosis (Seifert & Watkins, 1997).

Although it appears that osteoporotic bone loss is a result of an imbalance in the ratio of various localized regulators of bone turnover, there is some dispute as to whether this imbalance leads to uncoupling of resorption and formation or merely a change in the relative rates of the two processes. The issue appears to arise more as a result of differing definitions of "coupling" rather than different interpretations of the available data. If "coupling" is used to describe the sequential order of processes of the bone turnover cycle – ie resorption preceding formation then the general belief is that the two processes remain coupled in osteoporosis. If the definition of "coupling" is widened to include not only the sequential order of the two processes but also the synchronization of rates of formation and resorption so the net effect of a remodeling cycle is maintenance of bone mass then it can be said that uncoupling has occurred in osteoporosis.

1.6 Estrogen Imbalance - Bone Modeling

Case studies involving individuals with genetic defects in estrogen production or action provide evidence for a role of estrogen in promoting both the ordered sequence of the resorption and formation processes as well as the synchronization of the relative rates of bone resorption and formation.

Growth Hormone is believed to be the principle endocrine factor responsible for growth of long bones during childhood. In both males and females, estrogen has an important role in initiating and terminating the pubertal growth spurt. Low levels of estrogen promote Growth Hormone release and thereby indirectly accelerate the rate of long bone growth during puberty. High levels of estrogen induce closure of the epiphyseal growth plates thereby terminating bone modeling and initiating the coupled resorption/formation cycle of bone remodeling (Juul, 2001).

Three cases of men with genetic mutations resulting in a complete lack of estrogen or sensitivity to estrogen have been reported. In each case the individual failed to undergo a normal pubertal growth spurt and continued to grow throughout adulthood due to lack of

closure of the epiphyseal growth plates. Several cases of females with abnormally high estrogen levels have also been reported in which case the females were all of short stature due to early closure of the epiphyseal growth plates (Juul, 2001).

The observation that unsynchronized bone turnover occurs in estrogen deficiency suggests that maintenance of a high estrogen level is essential for synchronization of the two processes to occur regardless of whether the growth plates are closed.

1.7 Estrogen Imbalance - Bone Remodeling

The effects of estrogen imbalance on bone remodeling are many and varied. As mentioned in section 1.3.1, estrogen has an important role in controlling the relative levels of various localized regulators of bone metabolism. This impacts on the rates of osteoblastogenesis and osteoclastogenesis and also influences calcium balance in the body.

1.7.1 Effects of Estrogen on Osteoblastogenesis

Estrogen appears to restrict osteoblastogenesis as estrogen deficiency results in a temporal increase in the number of mesenchymal progenitor cells capable of differentiating to the osteoblast lineage as well as an increase in osteoblastic activity. During normal bone remodeling, osteoblast differentiation is initiated as factors present in bone matrix are released during osteoclast-mediated matrix degradation. Estrogen deficiency overrides this regulatory process by causing the synthesis and/or release of factors that promote osteoblastogenesis independent of the need created by bone resorption. The increased rate of osteoblastogenesis may partially be responsible for triggering the increase in osteoclastogenesis seen in estrogen deficiency (Jilka *et al*, 1998).

1.7.2 Effects of Estrogen on Osteoclastogenesis

Estrogen appears to down-regulate osteoclastogenesis via two mechanisms: direct suppression of RANK-L mediated osteoclast differentiation and indirectly through down-regulation of osteoclastogenic cytokines (Shevde *et al*, 2000). Ex-vivo bone marrow

cultures from ovariectomised mice exhibit a 3-4 fold increase in osteoclastogenesis compared to non-ovariectomised controls (Jilka *et al,* 1998).

Estrogen influences the synthesis and activity of various localized regulators of osteoclastogenesis such as pro-inflammatory cytokines through several mechanisms.

1.7.2.1 Oxidation

Estrogen exhibits antioxidant effects both directly through its A-ring hydroxyl group and indirectly via the cellular antioxidant enzyme system and by modulation of superoxide anion levels. Estrogen deficiency leads to an increase in free radical production which in turn induces pro-inflammatory cytokine expression in particular IL-1, IL-6 and TNF-α (Pfeilschifter *et al,* 2002). As noted in Section 1.3.2, many of these cytokines are localised regulators of bone metabolism.

1.7.2.2 Adiposity

Estrogen deficiency is associated with loss of bone mass as well as a gain in adipose tissue (Maeda *et al,* 2002). There is evidence that adipose tissue may be a major determinant of circulating IL-6 levels (Pfeilschifter *et al,* 2002).

1.7.2.3 Modulation of Cytokine Activity

The mechanism by which estrogen modulates cytokine activity is unknown but may involve interactions of the estrogen receptor with other transcription factors, modulation of nitric oxide activity, antioxidation, plasma membrane actions and/or changes in immune cell function (Pfeilschifter *et al,* 2002). Estrogen deficiency results in increased responsiveness of some cells to cytokines through modulation of synthesis of Specific Binding Proteins. Specific Binding Proteins are important in regulating cytokine activities by acting as decoys thereby competitively inhibiting cytokine receptor binding (Pfeilschifter *et al,* 2002). Estrogen also down-regulates the activity of other cytokines such as IL-6 which is involved in the signal transduction pathway leading to cytokine-mediated stimulation of simultaneous osteoblast and osteoclast development. Glycoprotein 130 (gp 130), the receptor subunit for IL-6, is inhibited by estrogen (Manolagus & Jilka,

1995). Several cytokines are capable of inducing the synthesis and release of prostaglandins from bone cells and associated tissues. Prostaglandins influence the synthesis and action of IGF which in conjunction with other bone growth factors is essential in the coupling of bone formation to resorption (see Section 1.4.2) (Watkins *et al,* 2001). TNF-α, IL-1, PGE_2 and IL-6 may have direct, RANK-L-independent stimulatory effects on osteoclastic differentiation (Pfeilschifter *et al,* 2002). TNF-α and IL-1 may exert their effects by enhancing stromal cell production of M-CSF, a potent stimulator of osteoclastogenesis (see Section 1.2.1) (Khosla *et al,* 1999). As very small amounts of RANK-L are required to maximally stimulate osteoclast formation it is possible that RANK-L independent mechanisms play a major role in the post-menopausal increase in osteoclastogenesis (Pfeilschifter *et al,* 2002).

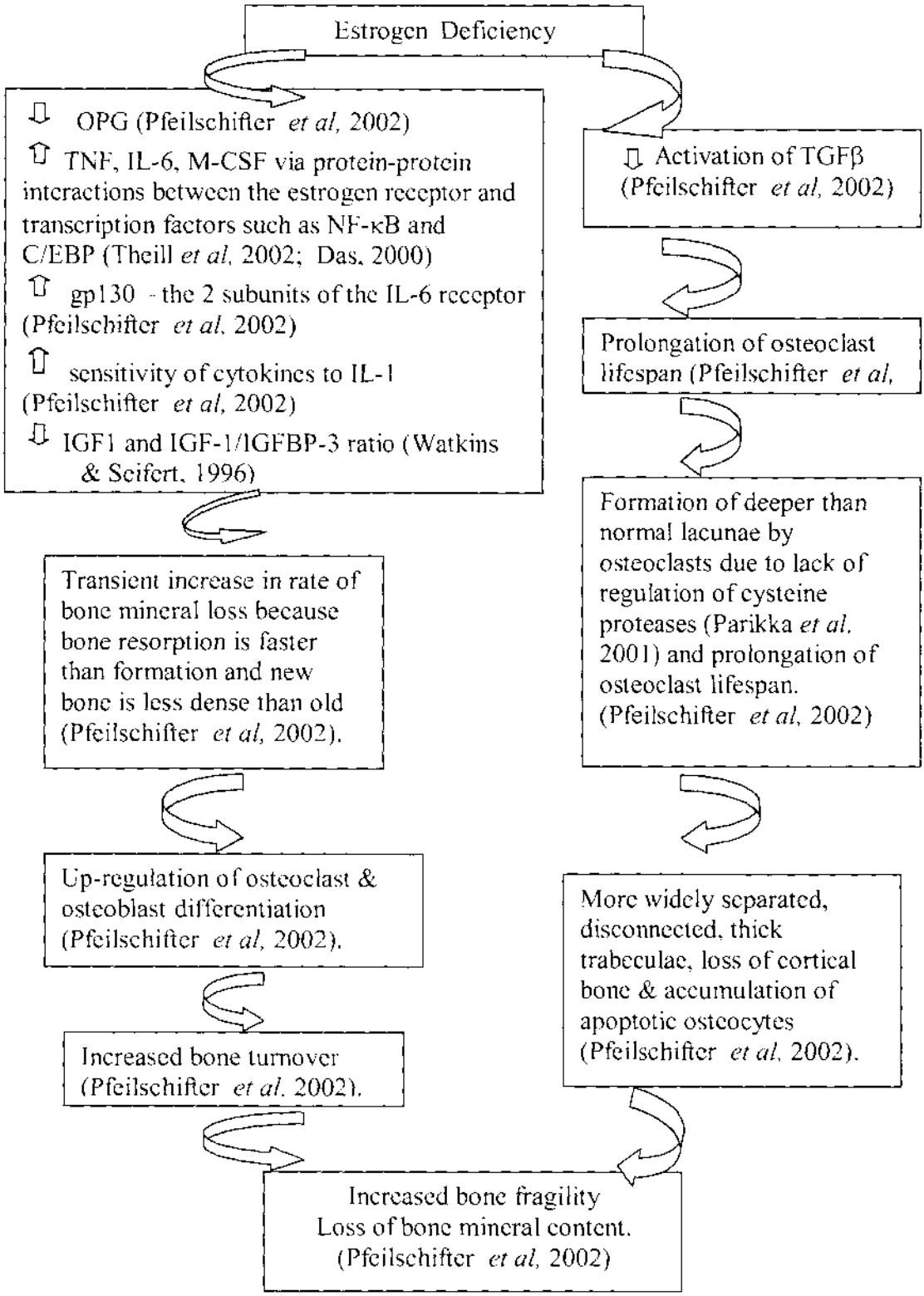
Localized factors may become more important regulators of bone metabolism post-menopause due to the reduced level of endocrine-mediated regulation as a result of estrogen deficiency. For instance, the effects of IL-11 on osteoclastogenesis are independent of estrogen status however the effects of IL-6 in promoting bone resorption are only apparent in a sex hormone deficient state (Manolagus & Jilka, 1995).

1.7.3 Effects of Estrogen on Calcium Absorption and Excretion

Estrogen deficiency decreases Ca^{2+} ATPase activity and increases vitamin D receptor availability (presumably as a result of reduced vitamin D receptor binding) resulting in decreased intestinal calcium absorption and increased urinary calcium excretion (Leonard *et al,* 2001). Other studies have demonstrated that estrogen increases vitamin D receptor number and bioresponse in the duodenal mucosa (Liel *et al,* 1999). It appears therefore that estrogen deficiency may result in decreased responsiveness of the body to vitamin D.

Age-related changes in hormone levels, gene regulation and cell responsiveness to hormones further compound the effect of menopause-induced estrogen deficiency. Levels of growth hormone (Nakamura *et al,* 2003), OPG (Makhluf *et al,* 2000) and rate of intestinal calcium absorption (Kruger & Horrobin, 1997) decrease with age whereas PTH levels increase (Seifert & Watkins, 1997).

Fig 8 Effects of estrogen deficiency on bone metabolism



SUMMARY

Estrogen is a key systemic regulator of bone turnover. During bone remodeling, it modulates the synthesis and activity of various localised regulators of bone metabolism such as cytokines and in so doing ensures the processes of resorption and formation are coupled. Estrogen deficiency as a result of natural menopause or ovariectomy leads to uncoupling of the two processes and a disproportionate increase in the rate of bone resorption compared to formation. The net result is a loss of bone mass. Age-related changes in the levels of other hormones such as growth hormone and PTH coupled with dietary factors such as inadequate dietary calcium and vitamin D intake and lifestyle factors such as low levels of load bearing exercise and sunlight exposure exacerbate the effects of estrogen deficiency further promoting bone mineral loss.

SECTION 3

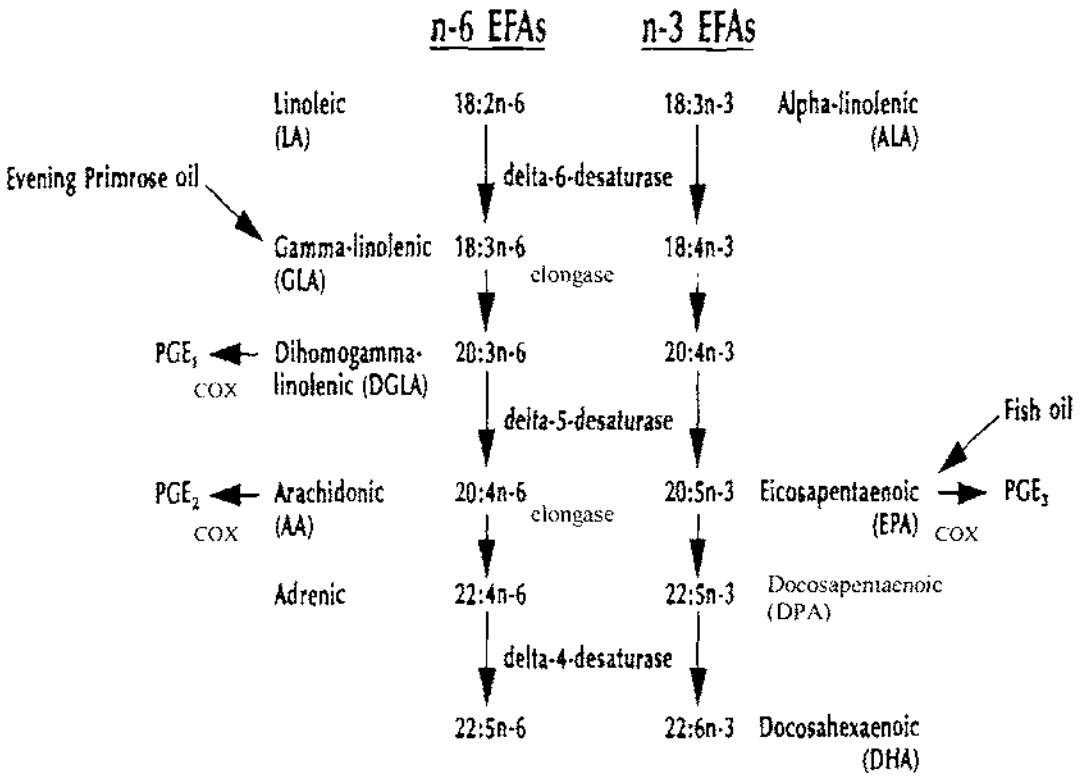
Essential Fatty Acids

Fatty acids are not only a source of energy for the body but they also serve as precursors for various other molecules, many of which are involved in the regulation of metabolism. Humans cannot synthesise fatty acids with a double bond past the carbon-9 position, therefore these unsaturated fatty acids are termed essential fatty acids as they must be obtained from the diet. Essential fatty acids (EFAs) are classified into one of two families designated as n-3 and n-6 depending on the location of the first unsaturated carbon from the methyl terminus (Fernandes *et al,* 2003). Alpha-linolenic acid (ALA) (18:3) is the parent compound for the n-3 series of fatty acids and linoleic acid (LA) (18:2) is the parent compound for the n-6 series. The most common dietary sources of n-3 EFAs are fish oil and green leafy vegetables where α-linolenic acid is present in chloroplasts. n-6 EFAs are present in many edible oils as linoleic acid is found in the seed oils of most plants (Albertazzi & Coupland, 2002).

1.8 Metabolism of Essential Fatty Acids

Both LA and ALA are metabolized by an alternating series of elongation/desaturation reactions. As shown in Figure 9, the same enzymes are responsible for metabolism of both n-3 and n-6 EFAs therefore ALA and LA compete with each other for enzyme binding. The ratio of ALA and LA in the diet is a major factor in determining the resultant mix of leukotrienes and prostaglandins produced.

Fig 9 Metabolism of Essential Fatty Acids



(Reproduced from van Papendorp et al. 1995)

1.8.1 Cyclooxygenase

Cyclooxygenase (COX) has greater specificity for arachidonic acid (AA) than eicospentaenoic acid (EPA) therefore COX preferentially synthesizes 2-series rather than 3-series eicosanoids (Watkins *et al,* 2001). There are two isozymes of COX. Cyclooxygenase 1 (COX-1) is predominately found in the endoplasmic reticulum and is constitutively expressed in most tissues. COX-2 is the inducible form of the enzyme - growth factors, hormones and cytokines stimulate its expression. n-6 fatty acids upregulate COX-2 expression and therefore promote 2-series prostaglandin formation. n-3 and saturated fatty acids inhibit COX-2 expression (Watkins *et al,* 2001). Estrogen stimulates COX-1 synthesis (Ospina *et al.* 2002) but appears to have little effect on COX-2 expression (Luo *et al.* 2001). Arachidonic acid is a potent stimulator of protein kinase C (PKC) and may up-regulate COX-2 by a PKC-mediated mechanism (Watkins *et al.* 2003). COX-2 is largely responsible for determining prostaglandin levels (Watkins *et al,* 2001).

1.8.2 Desaturases and Elongases

Delta-6-desaturase is the rate-limiting enzyme in EFA metabolism (James *et al*, 2003). Fatty acid desaturation is inhibited by smoking, diabetes, high sodium intake and biotin deficiency (Kruger & Horrobin, 1997). The activity of Δ-6-desaturase as well as Δ-5-desaturase reduces with advancing age. Corticosteroids, prescribed as a treatment of arthritis, also inhibit both EFA desaturases (Das, 2000). Calcium deficiency can inhibit essential fatty acid elongation (Kettler, 2001; Kruger & Horrobin, 1997).

1.9 Roles of Essential Fatty Acids and their Metabolites

1. Precursors for Eicosanoids (prostaglandins, leukotrienes etc).
   Eicosanoids regulate ion channels and ion transport (Kruger & Horrobin, 1997).
2. Regulation of Gene Expression
   Only EFAs which have undergone Δ-6 desaturation have the ability to regulate gene expression. The mechanism involves activation of the nuclear transcription factors known as PPARs (Peroxisome Proliferator Activated Receptors) by Δ-6 EFA desaturates. Once activated, PPARs form a heterodimer with another transcription factor known as the retinoid X receptor (RXR). The resultant complex binds to PPREs (Peroxisome Proliferator Response Elements) in the promoter regions of target genes (Stipanuk, 2000).
3. Regulate synthesis and secretion of cytokines eg IL-1, IL-6, TNF-α (Das, 2000).
4. May interact with statins promoting osteoblast proliferation and osteoclast apoptosis (Das, 2000).
5. Influence hydroxylation of vitamin D in the kidneys (Kruger *et al*, 1998).
6. Both vitamin D and EFAs cause changes in the distribution of phospholipids in the intestinal wall as well as increasing the degree of membrane unsaturation (Kruger *et al*, 1998).
7. Regulate cyclic nucleotide synthesis (Kruger & Horrobin, 1997).
8. Regulate protein kinase activity and therefore the phosphorylation and activation of various proteins (Kruger & Horrobin, 1997).
9. Regulate calcium signaling (Kruger & Horrobin, 1997).

1.10 Observed Relationship of Essential Fatty Acids and Bone

Lipids are essential for bone mineralisation as matrix from which lipids have been removed fails to mineralize (Kruger & Horrobin, 1997). Acidic phospholipids present in the bone matrix form complexes with calcium phosphate. This appears to be a crucial step in the mineralisation process and may explain the essentiality of lipids in this process (Mundy, 1995).

1.10.1 Epidemiological Studies

1.10.1.1 Essential Fatty Acid Deficiency

Both the amount and the type of fat in the diet have a direct effect on calcium balance in the body. In full term infants, the amount of fat in the diet is positively correlated with calcium absorption. Polyunsaturated fats in particular have been implicated as promoters of calcium absorption as incidence of tetany in formula-fed, full-term infants substantially declined when linoleic acid supplemented formula became available (Kruger & Horrobin, 1997). The mechanism may involve the action of vitamin D as the effect of vitamin D in stimulating calcium absorption and active calcium transport is considerably decreased in EFA deficiency (Kruger *et al,* 1998).

EFA deficiency also results in the deposition of calcium at abnormal sites within the body. EFA deficient animals exhibit increased renal and arterial calcification and develop severe osteoporosis (Das, 2000).

Postnatal skeletal development in rats is adversely affected by EFA deficiency as indicated by the increased occurrence of pathological fractures of long bones (Štěpánková *et al,* 1996). Loss of normal synthesis of bone connective tissue matrix, loss of normal cartilage, bone demineralization and replacement of bone with adipose tissue are associated with EFA deficiency (Kruger *et al,* 1998; Kruger & Horrobin, 1997).

1.10.1.2 Bone Density

Serum n-6 fatty acid levels have been reported as higher in individuals with bone loss compared to those without (Requirand *et al,* 2000). An imbalance between the ratio of n-

6:n-3 EFAs has been suggested as a cause of bone loss during adulthood (Horrobin *et al.* 2002).

Risk factors for low bone mineral density include smoking; low intakes of vitamin D, protein and calcium; high intakes of sodium; low BMI; diabetes; low levels of exercise and drug and alcohol use. Some of these factors also inhibit essential fatty acid elongation and desaturation (refer Section 1.8). It is possible therefore that the mechanism by which these factors cause low bone mineral density involves disruption of normal long-chain, fatty acid metabolism (Kruger & Horrobin, 1997).

1.10.1.3 Infants with Congenital Heart Disease

EFA metabolites have also been implicated as regulators of bone metabolism. IV infusion of the prostaglandin PGE_1 is used as a pre-operative treatment for infants with congenital heart disease in order to maintain the ductus in an open state pending corrective surgery. A side-effect of treatment is rapid bone growth (Kruger & Horrobin, 1997) implicating prostaglandins, particularly those derived from n-6 EFAs, as regulatory factors in bone metabolism.

1.10.1.4 Observational Studies

n-3 EFAs also appear to influence bone metabolism. Eskimos in Greenland consume a high n-3 fatty acid diet due to their high consumption of fish. The incidence of atherosclerosis, nephrolithiasis and osteoporosis is low among Greenland Eskimos compared to other countries (Das, 2000).

1.10.1.5 Kidney Stone Formation

Elevated levels of n-6 EFA metabolites such as arachidonic acid in plasma and erythrocyte phospholipids as well as elevated levels of PGE_2 are common in patients with idiopathic calcium nephrolithiasis indicating a n-6/n-3 EFA imbalance may promote kidney stone formation (Baggio *et al.* 2000). This indicates that the ratio of n-6/n-3 fatty acids may have a role in regulating calcium homeostasis and the site of calcium deposition within the body.

1.10.2 Clinical and Experimental Studies

1.10.2.1 Calcium Absorption and Excretion

Several studies have reported a positive effect of essential fatty acids on intestinal calcium absorption (Sabatini *et al,* 2002; Kruger & Horrobin, 1997). EFAs appear to have a direct effect on calcium absorption through the enhancement of membrane fluidity and may also have an indirect effect by facilitating vitamin D promotion of calcium uptake (Kruger & Horrobin, 1997). Studies in patients with pathological disturbances of the regulatory system responsible for maintaining calcium balance suggest that an imbalance between n-6 and n-3 EFAs and their metabolites may have a major role in the etiology of at least some of these conditions. One study involving idiopathic calcium oxalate stone formers reported a direct correlation between plasma PGE_2 and serum 1,25 $(OH)_2$ Vitamin D_3 levels. Calcium absorption, as measured by an oral strontium test, was closely correlated with the arachidonic acid content of phospholipid membranes. Supplementation with n-3 EFAs in the form of fish oil resulted in a decrease in the arachidonic acid content of membranes as well as in plasma PGE_2 levels and reduced the serum concentration of 1,25 (OH)2 Vitamin D_3 but not 25(OH) Vitamin D_3 to that of healthy controls. A reduction in intestinal calcium absorption also occurred most likely as a result of the decrease in concentration of calcium regulating hormones (Baggio *et al,* 2000).

Results of human studies on the effects of EFAs and prostaglandins on calcium excretion are conflicting. Levels of PGE_2 were positively correlated with both urine flow and calcium excretion in kidney stone formers and PGE_2 infusion resulted in increased calcium excretion (Kruger & Horrobin, 1997). Supplementation with either evening primrose oil (n-6 EFAs) or fish oil (n-3 EFAs) or a combination of both has been shown to decrease urinary calcium excretion in some but not all studies (Kruger & Horrobin, 1997). It is possible that gamma-linolenic acid (GLA) from evening primrose oil and EPA or docosahexaenoic acid (DHA) from fish oil increase renal calcium reabsorption (Kruger & Horrobin, 1997) however other factors such as dietary calcium, sodium or protein intake may mask this effect particularly in healthy individuals. Inhibition of prostaglandin synthesis or supplementation with fish oil has been shown to result in decreased urinary calcium excretion in rats (Kruger & Horrobin, 1997).

1.10.2.2 Bone Density – Human Studies

The ratio of n-3:n-6:saturated fatty acids in cortical bone and membranes and the concentration of circulating hexosamines (components of bone matrix proteins) is proportional to the ratio of these fatty acids in the diet (Watkins *et al,* 1997). It has been hypothesized that a high dietary ratio of n-6:n-3 fatty acids contributes to bone loss during maturity (Requirand *et al,* 2000).

Fish oil supplementation (4g per day, 16% EPA, 11% DHA) or a mixture of fish oil and evening primrose oil (4g per day, 60% linoleic acid, 8% GLA, 4% EPA, 3% DHA) for 16 weeks in elderly, osteoporotic women (mean age 80yrs) increased levels of serum calcium as well as levels of the bone formation marker osteocalcin. Alkaline phosphatase activity (which is associated with bone turnover) decreased and the ratio of urinary calcium/creatinine increased compared to subjects receiving 4g/day of placebo or evening primrose oil. The greatest increase in bone formation markers was achieved with the evening primrose oil/fish oil mixture (van Papendorp *et al,* 1995).

Supplementation of elderly, osteoporotic/osteopenic women (mean age 79.5 years) who had habitually low dietary calcium intakes with 6g of high EFA oil (60% LA, 8% GLA, 4% EPA and 3% DHA) in conjunction with 600mg calcium carbonate per day for 18 months resulted in decreased urinary phosphate excretion and maintenance of lumbar spine bone density compared to increased urinary phosphate excretion and a 3.2% decrease in lumbar spine density in subjects receiving 600mg calcium carbonate and 6g of coconut oil per day. Combined EFA/calcium supplementation for a further 18 month period resulted in an increase of 3.1% in lumbar spine bone density (Kruger *et al,* 1998). EFAs therefore appear to be beneficial in treating senile osteoporosis which is often caused by low dietary calcium intake coupled with a decreased ability to absorb dietary calcium and decreased vitamin D status as a result of lifestyle and metabolic factors associated with aging (refer Section 2.2) (Kruger & Horrobin, 1997).

One trial involving supplementation of pre and post-menopausal women (age range 25-40yrs and 50-65yrs respectively) with a combination of GLA, EPA and calcium for a

period of 12 months showed no additional benefit on bone density of EFA supplementation over calcium supplementation alone (Bassey *et al,* 2000). However the supplementation period was shorter and the EFA dosing regime (calcium 1g, evening primrose oil 4g and fish oil 440mg per day) was lower than that employed in trials reporting a positive effect of EFA supplementation. Confounding may have occurred due to other factors such as the subjects' saturated fat or zinc intake during the supplementation period. This trial also used total body DEXA measurements rather than lumbar spine and femur as used in other trials. Total body DEXA may lack the required sensitivity to detect changes in bone density due to EFA supplementation especially over the relatively short time frame of the trial period. Total body DEXA may also be a less accurate measure of bone density than regional DEXA due to interference from ectopic calcification (Demer, 2002). Women in this study were also considerably younger than those in the previous two reported studies and may have been at different stages of menopause.

The apparent dichotomy in the reported effects of essential fatty acid supplementation in postmenopausal women is probably a result of the confounding effects of aging.

1.10.2.3 Bone Density – Animal Studies

The majority of studies involving EFA supplementation have been conducted in animals.

1.10.2.3.1 Bone Modeling

In growing animals, feeding monounsaturated or n-3 or n-6 polyunsaturated fatty acids increases intestinal calcium absorption. However, an increase in calcium balance has only been observed when n-3 fatty acids are fed (Kruger *et al,* 1995). High dose supplementation of either n-6 or n-3 EFAs results in impaired bone formation during growth (Judex *et al,* 2000; Watkins *et al,* 2001*). One study reported a ratio of 1.2:1 n-6:n-3 EFAs resulted in the highest rate of bone formation during growth (Watkins *et al,* 2000). Another study reported a 3:1 ratio of GLA:EPA resulted in the lowest rate of bone resorption and highest bone calcium content in weanling rats (Watkins *et al,* 2000; Claassen *et al,* 1995). Low dose (0.5%) arachidonic acid supplementation led to increased bone mineral density in growing pigs but had no effect on calcium absorption or markers

of bone metabolism. This suggests that the essential fatty acids themselves are responsible for promoting intestinal calcium absorption but their metabolites have a role in stimulating bone mineralisation independent of rate of intestinal calcium absorption. It also appears that arachidonic acid is capable of increasing mineralisation of existing organic bone matrix independent of the rate of new matrix formation.

1.10.2.3.2 Bone Remodeling

The ovariectomised rat is used as a model for post-menopausal osteoporosis. Most of the studies conducted on EFA supplementation in mature animals have involved the use of ovariectomised rats with the aim of ascertaining any therapeutic effect of EFAs in promoting calcium balance and bone mineralisation in this pathological state. A combination of GLA, EPA and DHA fed to ovariectomised rats resulted in decreased D-Pyd excretion (a marker of bone resorption) and increased bone calcium content (Kruger *et al,* 1997). Supplementation with a GLA/EPA diester in conjunction with slow-release estrogen synergistically promoted bone mineralisation and inhibited resorption in ovariectomised rats (Schlemmer *et al,* 1999). In one study involving ovariectomised rats 160mg/kg body weight of EPA completely abolished the loss of bone weight in rats receiving a low calcium diet (0.01% calcium) but had no effect on bone weight in rats receiving a calcium-sufficient diet (1.25% calcium lactate) (Sakaguchi *et al,* 1994). EPA has been observed to increase the accumulation of ^{45}Ca in the cell layer in cultured osteoblastic cells and inhibition of PGE_2 has been demonstrated to stimulate calcification in cultured osteoblasts (Sakaguchi *et al,* 1994). 0.6g/100g of EPA or DHA reduced urinary phosphate losses and prevented the development of osteopenia in male, diabetic rats (Yamada *et al,* 1995).

Fish oil or EPA supplementation has been demonstrated to reduce deposition of calcium in kidneys and the aorta (Das, 2000).

The dietary balance of n-3 and n-6 fatty acids appears to be important in regulating both bone modeling and remodeling. The optimal dietary ratio of n-3 and n-6 EFAs for

maximising bone mineral density appears to differ in certain pathological states such as estrogen deficiency.

1.11 Mechanisms of Essential Fatty Acid Action in Bone

1.11.1 Membrane Structure

Arachidonic acid, eicosapentaenoic acid and docosahexaenoic acid are the most abundant long chain PUFA found in membrane phospholipids (Albertazzi & Coupland, 2002). Several studies have reported modulation of membrane and bone cell essential fatty acid levels by dietary fatty acid intake (Watkins *et al,* 1997; Atkinson *et al,* 1997; Watkins *et al,* 2000; Watkins *et al,* 1996). Oral supplementation of GLA or EPA results in increased incorporation of these fatty acids into intestinal membrane vesicles (Das, 2000). In one study, rats fed diets containing a 3:1 ratio of EPA:DHA at levels of 2.85 - 21.15% exhibited a 66-200% increase in total n-3 fatty acid and a 800-1940% increase in EPA levels in periosteum, marrow and cortical bone polar lipids. PGE_2 production fell in proportion to the decreased ratio of AA/EPA in bone tissue polar lipids. A disproportionately high level of EPA compared to DHA was incorporated into polar lipids however the level of DHA and EPA incorporated into neutral lipids was similar to controls (Watkins *et al,* 2000).

Supplementation of EFA-deficient chicks with linoleic acid resulted in increased calcium uptake in isolated vesicles which was correlated with the amount of linoleic acid in the brush border membrane (Kruger & Horrobin, 1997).

Vitamin D supplementation of vitamin D deficient chicks resulted in increased incorporation of arachidonic acid into phosphatidylcholine as well as increased incorporation of phosphatidylcholine and phosphatidylethanolamine into brush border membranes. The rapid increase in transmembrane calcium transport in response to vitamin D administration may occur due to a vitamin D-promoted increase in intestinal phospholipase A2 activity followed by acyltransferase-mediated reacylation resulting in formation of phoshatidylcholines with a different fatty acid composition (Kruger &

Horrobin, 1997). EFAs may therefore act in conjunction with vitamin D to alter the lipid content of membranes thereby influencing calcium absorption and reabsorption.

There is some evidence that acidic phospholipids act as non-energy-requiring calcium "traps" (Watkins & Seifert, 1996). Therefore a high EFA diet leading to increased membrane incorporation of EFAs may promote calcium binding to membranes. Dietary EFA intake may influence the fatty acid composition of the osteoblast phospholipid membrane and as a consequence, the matrix vesicle membrane which may influence the affinity of the membrane constituents for calcium binding (Watkins & Seifert, 1996).

Membrane fluidity decreases with age and may account for the observed age-related decrease in V_{max} for calcium uptake (Kruger & Horrobin, 1997). It is possible that increased incorporation of EFAs into the brush border membrane helps negate the age-related decrease in membrane fluidity and hence may promote calcium uptake. Membrane structure changes may also influence the conformation and therefore activity of membrane associated enzymes.

1.11.2 Activity of Membrane Transporters

Activity of various membrane-associated proteins such as Na^+K^+-ATPase, Ca^{2+}-ATPase, Ca^{2+}-transferase and Mg^{2+}-ATPase is dependent on the amount and type of fatty acid consumed (Leonard *et al*, 2001). In rats, mucosal transference of Ca^{2+} was found to decrease with ovariectomy by 11.4-14.3%. High dietary fat intake (30%) in ovariectomised rats was shown to reduce membrane-associated enzyme activity (5.0-15.9%) with the greatest reduction occurring with a high saturated fat diet (11.02-15.94%) compared to a high monounsaturated (9.1-14.8%) or polyunsaturated (5.0-12.1%) fat diet (Chanda *et al*, 1999). Supplementation of ovariectomised rats with a 1:3 compared to a 3:1 ratio of GLA/EPA+DHA resulted in the greatest increase in Ca^{2+}ATPase activity and also decreased the number of available vitamin D receptors (Leonard *et al*, 2001). Conversely supplementation with a 3:1 ratio of GLA/EPA+DHA increased intestinal calcium absorption in weanling male rats by 47% whereas a 1:3 ratio only resulted in a 21.4% increase in absorption. Bone calcium content remained at baseline levels in the 3:1 group

but declined in the 1:3 group (Claassen *et al,* 1995). A similar effect was found by the same researchers in a mature rat model (Claassen, 1996). All three studies involved feeding a low calcium diet. The apparent discrepancy between studies may indicate there is a greater requirement for dietary n-3 fatty acids in instances of estrogen deficiency. However all three studies reported either the EFA composition of plasma or erythrocyte membranes and differences were evident particularly in the ratio of EPA:AA. In both the Claassen *et al,* 1995 and Claassen, 1996 studies where greater Ca^{2+}-ATPase activity was found with a 3:1 ratio of GLA:EPA+DHA the ratio of EPA:AA in plasma lipids was 0.34 in the 1:3 group and 0.04 in the 3:1 group. In the Leonard *et al,* 2001 study which reported greater Ca^{2+}-ATPase activity with the 1:3 GLA:EPA+DHA supplement, the ratio of EPA:AA in erythrocyte membranes was 0.14 in the 1:3 group and 0.014 in the 3:1 group. It is possible therefore that the ratio of EPA:AA in membranes is important for Ca^{2+}-ATPase activity and that high levels of EPA inhibit Ca^{2+}-ATPase activity. As discussed in section 1.3.2, one of the mechanisms by which 1,25$(OH)_2$ vitamin D_3 increases intestinal calcium absorption may be via increasing the AA content of membranes (Baggio *et al,* 2000; Baggio, 2002; Baggio *et al,* 2000*). The finding that vitamin D receptor number was decreased as the ratio of EPA+DHA:GLA increased may indicate that n-3 fatty acids act as feedback inhibitors of vitamin D activity.

Arachidonic acid inhibits Na^+-K^+-ATPase (Haag *et al,* 2001) and Mg^{2+}-ATPase (Haag *et al,* 2000) in a dose dependent manner and activates Ca^{2+}-ATPase (Haag & Kruger, 2001). Unsaturated fatty acids interact with the S2-S3 loop of Ca^{2+}-ATPase and appear to mimic the action of calmodulin thereby removing auto-inhibition of the enzyme caused by interaction between its S2-S3 and S4-S5 cytosolic loops (Haag & Kruger, 2001). Polyunsaturated fatty acids activate protein kinases A and C and may stimulate Ca^{2+}-ATPase indirectly by promoting phosphorylation of the enzyme (Haag & Kruger, 2001) however the mechanism by which arachidonic acid inhibits Na^+-K^+-ATPase and Mg^{2+}-ATPase does not appear to involve protein kinase C (Haag *et al,* 2000; Haag *et al,* 2001).

PGE_2 modulates renal calcium handling by inhibiting the Na/K/2Cl co-transporter. It promotes calcitriol formation thereby enhancing intestinal calcium absorption and

inhibiting renal calcium reabsorption (Baggio *et al*, 2000). Arachidonic acid inhibits K^+ currents in osteoblasts. At low levels arachidonic acid promotes calcium entry into osteoblasts but at high levels it inhibits calcium uptake (Kruger & Horrobin, 1997).

1.11.3 The Role of Eicosanoids

Eicosanoids modulate the rate of bone formation (Watkins *et al*, 1996). Dietary polyunsaturated fat intake influences the amount and type of eicosanoids formed (Watkins *et al*, 2001*). This is demonstrated by the results of the following studies. Rats supplemented with 70g of n-6 or n-3 fatty acids/kg body weight had high tissue levels of n-6 or n-3 fatty acids respectively. A high n-6 fatty acid diet resulted in increased PGE_2 production (Li *et al*, 1999). Supplementation of male rats with 10g/kg body weight of conjugated linoleic acid (CLA) resulted in increased membrane concentration of CLA as well as of saturated and n-3 fatty acids in bone tissue, marrow, liver, serum and periosteum and decreased membrane incorporation of n-6 fatty acids and production of PGE_2. The latter is possibly a result of competitive inhibition by CLA of Δ9-desaturase and n-6 fatty acid metabolism as 18:1 n-9 tissue concentration decreased and level of total saturates increased with CLA supplementation (Li & Watkins, 1998).

1.11.3.1 Prostaglandins

Prostaglandins are a type of eicosanoid and are believed to be one of the major factors responsible for localised control of bone metabolism. Little is known about the functions of many of the other eicosanoids in bone metabolism but there has been considerable research focus on the roles of prostaglandins.

Increased n-3 fatty acid consumption results in a switch from PGE_2 synthesis by cyclooxygenase to synthesis of PGE_3 and 5-series leukotrienes (Seifert & Watkins, 1997). PGE_3 is at least as potent as PGE_2 in stimulating bone resorption however EPA is a less effective substrate for cyclooygenase than arachidonic acid therefore the net effect of increased EPA/AA is a decrease in the levels of bone resorbing prostaglandins (Watkins *et al*, 2000). Leukotrienes are potent stimulators of bone resorption but also enhance osteoblast proliferation thereby promoting bone formation (Seifert & Watkins, 1997).

Estrogen deficiency enhances the localized production of inflammatory cytokines, potentiates PGE_2 production and increases the susceptibility of bone tissue to the catabolic effects of cytokines (Watkins *et al,* 2001*). Physical stress, PTH, EGF (Erythrocyte Growth Factor), PDGF, TGF and interleukins stimulate prostaglandin synthesis and release in osteoblasts (Seifert & Watkins, 1997). Protein kinase C activation may also stimulate PGE_2 synthesis (Leis *et al,* 1998).

Prostaglandins are believed to initiate bone resorption via mediation of the anabolic effects of biomechanical forces, parathyroid hormone, cytokines eg TNF-α and IL-3; growth factors such as TGF-β, PDGF, bFGF and insulin-like growth factors and IGF binding proteins 3 and 5 (Watkins *et al,* 1997; Watkins *et al,* 2001#; Seifert & Watkins, 1997).

PGE_2 is generally considered one of the major factors responsible for the osteoclastogenic effect observed as a result of a high dietary ratio of n-6/n-3 fatty acids during maturity (Watkins *et al*, 2001*). PGE_2 is involved in initiating bone resorption and the subsequent mobilization of stored calcium. Its action is dose dependent as low levels (10^{-10} – 10^{-8} M) of PGE_2 stimulate bone formation whereas high levels (10^{-6} M) inhibit bone matrix formation and suppress osteoblast differentiation in growing rats (Li *et al,* 1999; Watkins *et al*, 2001#).

Exogenous PGE_2 administered via subcutaneous injection (1mg - 6mg PGE_2/kg/day) has been shown to stimulate bone growth at cortical and trabecular bone sites in mature ovariectomised rats to a greater extent than in young rats (Ke *et al,* 1993; Ma *et al,* 1994). This may indicate that not only does estrogen deficiency increase the responsiveness of bone cells to cytokines (Watkins *et al,* 2001*) but it may also increase their responsiveness to eicosanoids.

1.11.3.2 Interactions between Essential Fatty Acids, Eicosanoids and Hormones

EFAs may modulate the interaction of estrogen with its receptor. Abnormal levels of EFAs and/or their metabolites may result in sub-optimal responses to estrogen (Das, 2000).

PGE_2 may be the primary means by which PTH and 1,25-$(OH)_2$ vitamin D_3 exert their osteoclastogenic effects (Watkins *et al,* 2001*). PTH levels are commonly elevated in the elderly. One study showed elderly women supplemented with a GLA/EPA mixture had normal PTH levels whereas subjects receiving a placebo had typically elevated PTH levels. Some arachidonic acid metabolites can inhibit PTH secretion (Kruger & Horrobin, 1997).

1.11.3.3 Interactions between Essential Fatty Acids, Eicosanoids and Localized Regulatory Factors

Interleukins 1 and 6 together with TNF-α appear to be the major localized regulators of bone catabolism involved in the development of osteoporosis. Estrogen exhibits negative feedback control on IL-1 and TNF-α secretion by peripheral blood monocytes and inhibits secretion of IL-6 by IL-1 or TNF-activated stromal cells and osteoblasts (Das, 1994). PGE_2 stimulates the release of IL-6 possibly indirectly via IL-1β. IL-1β induces COX-2 expression therefore further increasing PGE_2 levels (Kruger & Horrobin, 1997). Arachidonic acid may have a much greater stimulatory effect on cytokine expression than PGE_2 as treatment of osteoblast cells with arachidonic acid in the presence of a cyclooxygenase inhibitor did not interfere with the observed induction of cytokine expression (Baggio, 2002).

A low fat diet containing a high proportion of fish oil (equivalent to 1.23g/day EPA and DHA) was shown to decrease levels of inducible IL-1β, IL-6 and TNF-α by 40%, 34% and 35% respectively compared to baseline in middle-aged men and women. In comparison a low fat, low fish oil diet (equivalent to 0.27g/day EPA and DHA) resulted in a significant increase in inducible IL-1β and TNF-α levels (62% and 47% respectively) (Kettler, 2001).

n-3 essential fatty acids increase production of nitric oxide via the constitutive pathway and decrease inducible nitric oxide production. Activation of the inducible Nitric Oxide Synthase pathway is associated with increased osteoclastic activity (Watkins *et al,* 2003).

Essential fatty acids may decrease c-AMP by the action of secondary messenger systems (Kruger *et al,* 1997.) This may reduce the osteoclastogenic effect of PGE_2 by inhibiting the EP2 and EP4 prostaglandin receptor signaling pathways.

The age-related reduction in growth hormone levels signals a reduction in the rate of bone formation through decreased IGF-1 expression (Watkins *et al,* 2001*). Estrogen deficiency also decreases IGF-1 levels (Watkins & Seifert, 1996). This, combined with the age-related decrease in responsiveness of osteoprogenitor cells to IGF-1, leads to decreased bone formation (Tanaka & Liang, 1996). One mechanism by which essential fatty acids may exert their effects on bone metabolism is by promoting the synthesis of IGF-1. Low concentrations of PGE_2 increase IGF-1 transcription and translation possibly due to enhancement of c-AMP levels (Watkins & Seifert, 1996). Polyunsaturated fatty acids may enhance IGF-1/receptor interaction by increasing membrane fluidity. PGE_2 also promotes transcription of selective IGF-binding proteins such as IGFBP-3, IGFBP-4 and IGFBP-5 and enhances the binding affinity of IGFBP-3 (Watkins & Seifert, 1996). IGFBP levels are increased in rats fed a CLA/n-6 fatty acid supplement and decreased in rats fed a CLA/n-3 fatty acid supplement (Li *et al,* 1999). The balance of essential fatty acids in the diet is important however as high levels of PGE_2 inhibit bone formation by reducing IGF-1 levels (Watkins *et al,* 2001*).

Other EFA metabolites such as leukotrienes are also involved in regulation of bone metabolism and calcium balance within the body. Lekotriene B4 (LTB4) may interact with prostaglandins to regulate osteoblast activity. LTB4, PTH and PGE_2 activate the breakdown of phospholipids in osteoblast membranes thereby increasing the ratio of bone resorption to formation (Seifert & Watkins, 1997). LTB4 stimulated bone resorption to a greater extent than PGE_2 in mouse calvaria (Watkins *et al,* 2000). EPA decreases production of LTB4 and thromboxane B2 (TxB2) and increases Leukotriene B5 (LTB5), a lipoxygenase product of EPA. LTB5 may exhibit similar effects to PGE_2 in stimulating bone resorption as well as formation. LTB5 is less bioactive than LTB4 (Watkins *et al,* 2000).

It is likely that dietary EFA balance is important for determination of the relative ratio of eicosanoids within the body which in turn are involved in the cytokine-mediated maintenance of bone homeostasis.

1.11.4 Regulation of Osteoclast/Osteoblast Differentiation

An age-related decrease in osteoblast number per unit bone length has been observed in rats however pre-osteoblast, pre-osteoclast and osteoclast cell numbers appear to be independent of age (Roholl *et al,* 1994). The decreased differentiation of pre-osteoblasts may be due to the observed age-related deficit in the response of osteoprogenitor cells to IGF-1 (Tanaka & Liang, 1996).

Peroxisome Proliferator-Activated Receptor – γ (PPAR-γ) is a nuclear transcription factor expressed on osteoblasts which promotes differentiation into adipocytes (Corwin, 2003) (refer Section 3.2). Activation of PPAR-γ by saturated fatty acids as well as linoleic acid peroxidation products has been shown to induce osteoblast differentiation into adipocytes and inhibit osteoblastogenesis in vitro (Diasero *et al,* 1998). A diet low in saturated fatty acids and rich in antioxidising agents therefore may promote osteoblastogenesis.

EFAs and their metabolites are potent inhibitors of HMG-CoA reductase, the enzyme catalyzing mevalonate synthesis. Isoprenoid precursors such as mevalonate are required for activation of Ras and various other small GTPases via phenylation. Some of these small GTPases appear to inhibit BMP-2 expression leading to decreased osteoblast formation (Das, 2000).

IL-1 and TNF-α production is inhibited by essential fatty acids and their metabolites eg arachidonic acid, EPA and DHA (Das, 2000). Arachidonic acid and possibly other EFA metabolites also inhibit nuclear translocation of NF-κB (Das, 2000). IL-1, TNF-α and NF-κB are all involved in promoting osteoclastogenesis therefore EFAs have an inhibitory effect on osteoclastogenesis.

n-3 EFAs may decrease the RANK-L/OPG ratio thereby inhibiting osteoclastogenesis. However osteoclast formation is not inhibited by EPA (Watkins *et al*, 1997) therefore other n-3 EFAs or n-3 EFA metabolites must be responsible for this effect. n-3 EFAs may also upregulate Cbfa1 expression thereby promoting osteoblastogenesis (refer Section 1.2.3) (Watkins *et al*, 2001#).

PGE_2 may stimulate osteoclastogenesis either directly or indirectly through inhibition of OPG expression and promotion of RANK-L expression (Watkins *et al*, 2001*).

1.11.5 Collagen Synthesis

The IGF-1/IGF-binding protein axis may have an important function in collagen and proteoglycan synthesis. Low levels of PGE_2 and high concentrations of cAMP both activate the IGF-1/IGF binding protein axis which stimulates collagen synthesis. High levels of PGE_2 promote the synthesis of inflammatory cytokines leading to net loss of proteoglycan from cartilage. (Watkins *et al*, 2000).

Collagen synthesis decreased in chick epiphyseal chondrocytes cultured on a linoleic acid-enriched growth medium but increased when n-3 EFA or CLA-enriched media was used. EPA enrichment of growth medium also resulted in enhanced collagen synthesis by fibroblasts compared to arachidonic acid-enriched medium (Watkins *et al*, 2001). This effect is probably a result of reduced PGE_2 synthesis due to EPA treatment.

Inflammatory cytokines eg IL-1 inhibit chondrocyte proliferation and induce cartilage degradation. n-3 fatty acids decrease the proteolytic activity of aggrecanase and collagenase (Watkins *et al*, 2003) which may explain the observed beneficial effect of n-3 fatty acids in the treatment of inflammatory joint diseases such as osteoarthrosis and rheumatoid arthritis (Watkins *et al*, 2001*). It is unknown however whether all n-3 fatty acids are equally potent in inhibiting the activities of these enzymes.

Feeding n-6 or n-9 EFAs to growing chicks did not increase the levels of these fatty acids in epiphyseal cartilage, chondrocytes or matrix vesicles. Consumption of n-3 fatty acids

however resulted in increased levels of these fatty acids in cartilage (Seifert & Watkins, 1997). This suggests that cartilage selectively incorporates certain dietary fatty acids during longitudinal bone growth and may indicate that n-3 fatty acids have a regulatory role in cartilage metabolism.

SUMMARY

The ratio of n-3/n-6 EFAs required to optimize calcium balance and bone density differs depending on the physiological state of the individual. Pathological conditions such as those associated with estrogen deficiency or kidney stone formation may arise either directly or indirectly from an EFA imbalance. EFA supplementation may therefore aid in correcting these conditions.

Various EFA supplementation regimes have been shown as beneficial in promoting calcium absorption, reducing urinary phosphate excretion as well as in modulating the rate of bone resorption compared to formation leading to increased bone density. Different n-3 and n-6 EFAs eg EPA vs DHA and GLA vs arachidonic acid, may exert different effects on bone turnover. The optimal ratio of n-3:n-6 EFAs and the effects of different members of each EFA family on bone metabolism are unknown.

Essential fatty acids exert their effects on calcium balance and bone metabolism by several different mechanisms.

1) **Intestinal calcium absorption** EFAs and their metabolites act in two different ways to enhance intestinal calcium absorption. Firstly dietary EFAs directly influence membrane structure. They may negate the effect of ageing on diminishing intestinal calcium absorption by increasing the level of unsaturated fatty acids in membrane phospholipids. This may facilitate passive uptake of calcium and/or up-regulate the activity of membrane-associated calcium transporters. EFAs may also facilitate the action of vitamin D in promoting calcium absorption. Vitamin D acts to increase the incorporation of EFAs into intestinal membranes leading to increased calcium absorption. As vitamin D levels are often reduced in the elderly,

increased dietary EFA intake may be a means of compensating for the lack of vitamin D activity. Secondly EFA metabolites such as prostaglandins mediate the activity of membrane-associated ion transporters thereby increasing active calcium uptake.

2) **Renal calcium excretion** – PGE_2 acts in two ways to inhibit renal calcium reabsorption. It promotes the formation of calcitriol from vitamin D and inhibits the $Na/K/Cl_2$ co-transporter. n-3 EFAs and/or their metabolites may inhibit $1,25(OH)_2$ vitamin D_3 formation and promote reabsorption of phosphate in the renal tubules.

3) **Osteoclastogenesis** - n-3 EFAs (except EPA) inhibit osteoclastogenesis by modulating the RANK-L/OPG ratio. This may occur by competitive inhibition of PGE_2 formation (a promoter of RANK-L expression) and/or via modulation of the levels of cytokines such as IL-1.

4) **Osteoblastogenesis** – EFAs may promote osteoblast formation by modulating the synthesis or activation of inhibitors of osteoblastogenesis. n-3 EFAs may promote osteoblast formation by increasing levels of transcription factors such as Cbfa1.

5) **Collagen synthesis** – n-3 and n-6 EFAs appear to have opposing roles in the regulation of collagen synthesis. Whereas n-3 EFAs promote collagen synthesis and inhibit proteolytic collagen degradation, in high concentrations the n-6 metabolite PGE_2 inhibits collagen synthesis and promotes its breakdown. Promotion of collagen synthesis by n-3 EFAs may result from competitive inhibition of PGE_2 synthesis. n-3 EFAs inhibit collagen degradation both by competitive inhibition of PGE_2 synthesis as well as by reducing the activity of catabolic enzymes.

Essential fatty acids appear to act by a wide range of mechanisms to regulate calcium balance and subsequently, bone metabolism.

SECTION 4

Motivation and Objectives

1.12 Motivation for the Study

It has been established that EFAs have a role in the regulation of bone metabolism particularly in instances of estrogen deficiency (Kruger & Horrobin, 1997). However, insufficient information is yet available to enable the development of specific dietary recommendations for the prevention and/or treatment of osteopenia and osteoporosis (Brown *et al,* 2002). Whilst it is generally recognized that the ratio of n-3:n-6 EFAs is important in regulating bone turnover (Watkins *et al,* 2001), the optimum ratio for maximising bone mass has yet to be determined. There is some indication that specific EFAs in each family may have different roles in the regulatory process and synergism between specific EFAs rather than just the between the EFA families may be the means by which regulation occurs (Horrobin *et al,* 2002). To date there are few reports as to the effects of specific EFAs on bone turnover. In order to eventually provide dietary recommendations or develop effective neutraceuticals to aid in combating post-menopausal bone loss, the roles of specific EFAs and their interaction with one another needs to be elucidated.

Various studies have demonstrated a beneficial effect of fish oil or combinations of GLA, EPA and DHA on bone density in ovariectomised rats as well as postmenopausal women (Kruger *et al,* 1997; Kruger *et al,* 1998; Kruger *et al,* 1999; Schlemmer *et al,* 1999, van Papendorp *et al,* 1995). EPA is the main EFA in fish oil. The studies which have used a combination of specific EFAs have generally concluded a high ratio of EPA and DHA:GLA is most beneficial in increasing bone mass (Kruger *et al,* 1997; Kruger *et al,* 1998; Kruger *et al,* 1999; Schlemmer *et al,* 1999, van Papendorp *et al,* 1995). *In vitro* studies have reported greater calcium sequestering in EPA-treated osteoblasts compared to non-EPA treated controls (Sakaguchi *et al,* 1994). EPA supplementation of ovariectomised rats has been reported to increase bone strength compared to ovariectomised controls (Sakaguchi *et al,* 1994).

EFAs have been implicated as possible mediators of vitamin D_3 action and fish oil supplementation has been associated with elevated 25(OH) vitamin D_3 concentrations (Baggio *et al.* 2000). As vitamin D_3 is a key systemic regulator of bone turnover and sensitivity to $1,25(OH)_2$ vitamin D_3 is reduced in estrogen deficiency (Leonard *et al.* 2001), this may also have important consequences for bone density.

1.13 Purpose of the Study

From the literature it appears that EPA may have a beneficial role in combatting the reduction in bone mass resulting from estrogen deficiency. The aim of the present study was to focus on the specific effect (if any) of EPA on bone turnover.

1.14 Objectives

The specific objectives of this study were to determine the effect of EPA supplementation in ovariectomised rats on:

- bone density
- rate of bone resorption
- bone strength

Secondary objectives were to determine if EPA supplementation altered vitamin D_3 metabolism and to ascertain the effects of EPA administration on plasma lipid composition.

1.15 Hypothesis

EPA supplementation will prevent bone loss due to ovariectomy.

CHAPTER 2

MATERIALS AND METHODS

2.1 Procedures

2.1.1 Animals

Sixty 7-month old female Sprague-Dawley rats were obtained from the Small Animal Production Unit, Massey University. The animals were sham-operated or ovariectomised (OVX) at age 8 months (week 0 of the study). Sham operated animals (n=15) were anaesthetised and an incision made but the ovaries left intact. The ovaries were removed from the OVX animals and these animals were randomised into 3 groups.

The animals were separately housed in shoebox cages, and kept in a temperature- (22°C ± 2°C) and light-controlled (12 hour day/night cycle) room in the Small Animal Production Unit (Massey University). Animals had *ad libitum* access to deionised water. The trial ran for 10 weeks. Massey University Animal Ethics Committee approved the study (03/18).

2.1.2 Diets

The animals were fed a balanced semi-synthetic diet consisting of 14% caseinate, 5% cellulose, 4% corn oil, 0.5% calcium, 60% starch and added vitamins and minerals as needed commencing four weeks prior to the ovariectomies. After the animals were ovariectomised or sham operated they were either maintained on the control diet (SHAM (n=15) and OVX Control (n=15 at baseline)) or fed a diet supplemented with EPA (refer Table 2 for specification). The EPA-treated animals received either 1.0g EPA/kg body weight/day (high dose, n=15) or 0.1g EPA/kg body weight/day (low dose, n=15 at baseline). Corn Oil was added to the EPA-supplemented diets so total oil content of each diet was 4%. Corn oil is a source of n-6 essential fatty acids and was used in order to prevent n-6 fatty acid deficiency in the study animals. A minimum of 1% corn oil is required in order to prevent deficiency and diets contained no less than 1.5% corn oil. The

daily food intake of the animals was measured, and the amount of EPA added to the feed for supplemented animals was adjusted weekly according to the mean body weight of animals in each treatment group. Sham animals were fed *ad libitum*. The food intake for ovariectomised animals was limited to that of the sham controls in order to reduce OVX-induced weight gain.

Table 2 Specification for EPA supplement

EPA ethyl ester	Min 96% (actual tested 99%)
A-tocopherol	0.2%
Arachidonic Acid	<1%
Docosahexaenoic Acid	<1%
Docaspentaenoic Acid	<1%
Density	0.91
Acid Value	<1
Iodine Value	~ 370
Total Heavy Metals	<10ppm
Arsenic	<2ppm
POV	2meq/ÜL

2.1.3 Tail-Vein Sampling

At week –2 rats were placed in a purpose-built restrainer, which was then placed on top of a heat pad under a heat lamp. A tourniquet was placed around the base of the tail. 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 3000 rpm for 10 minutes. The plasma was removed, snap-frozen with liquid Nitrogen, and then stored at –85 °C.

2.1.4 Dual Energy X-Ray Absorptiometry (DEXA) Scans

Animals were scanned for baseline measurements at week –2. Repeat scans were conducted *in vivo* at week 4 and week 10. *Ex vivo* measurements were made at week 11.

Animals were weighed and anaesthetised with an appropriate dose of anaesthetic ie. 0.05ml/100g body weight. The anaesthetic was a mixture of 0.2ml Acepromazine (ACP) + 0.5ml Ketamine + 0.1 Xylazine + 0.2ml sterile H_2O, and was administered via an intra-peritoneal injection using a 25G x 5/8" needle and 1ml syringe. The animals attained a suitable level of anaesthesia approximately five to ten minutes after injection and remained under anaesthesia for up to 2 hours.

Bone mineral measurements were made with a Hologic QDR4000 bone densitometer using a pencil beam unit (Bedford, USA). A daily Quality Control (QC) scan was taken to ensure precision met with the required coefficient of variation. Regional high-resolution scans were performed using a 0.06" diameter collimator with 0.0127" point resolution and 0.0254" line spacing. Rats were placed on an acrylic platform of uniform 1.5" thickness. Each rat underwent three regional high-resolution scans of the spine and left and right femurs. Rats were positioned supine with right angles between the spine and femur, and femur and tibia.

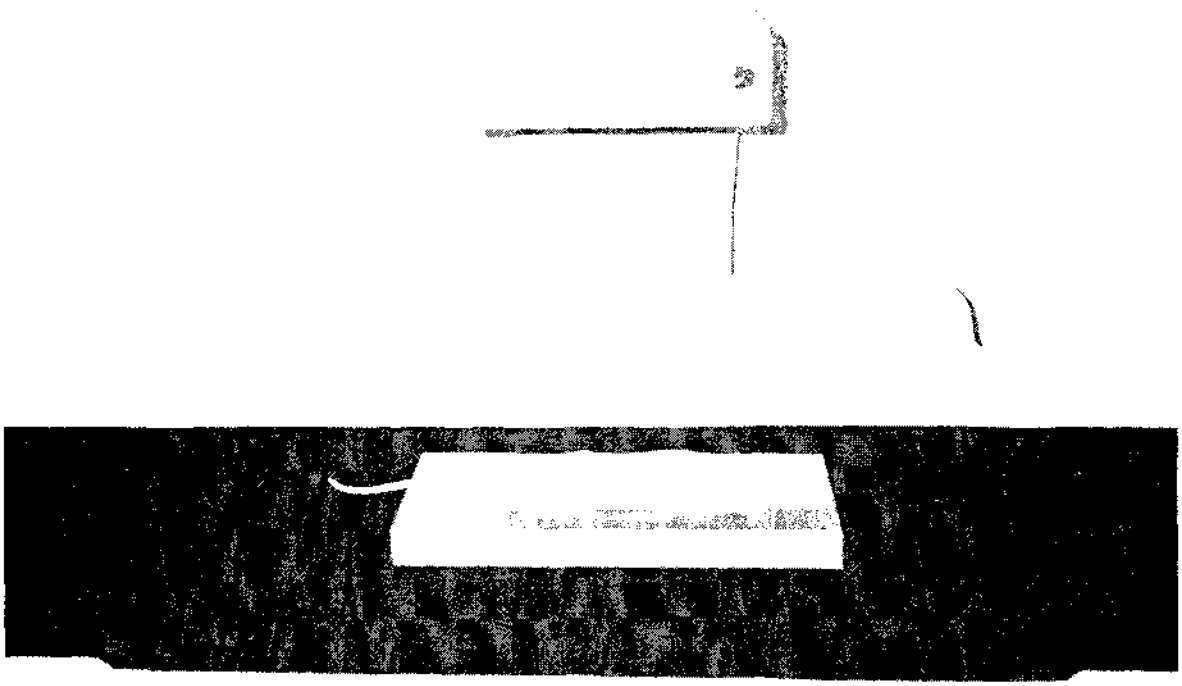


Fig 10 Photograph showing positioning of a rat for DEXA scanning.

2.1.5 Terminal Heart Puncture

At week 10, animals were weighed and anaesthetised with an appropriate dose of anaesthetic ie. 0.1ml/100g body weight. A 19G x 1 ½" needle and 5ml syringe were used to withdraw blood directly from the heart. Blood samples were collected into vacutainers containing heparin, then centrifuged at 3000 rpm for 10 minutes. The plasma was removed, snap-frozen with liquid nitrogen, and then stored at –85 °C.

Animals were then euthanased under anaesthesia by carbon dioxide inhalation, and dissected. Both the right and left femur and spine were excised with some flesh remaining and frozen in phosphate-buffered saline (PBS) for further analysis. The uteri were removed and weighed to confirm ovariectomy has been successful.

2.1.6 Urine Collection

One day prior to euthanasia, rats were placed in metabolic cages overnight (4pm - 8am). Urine was collected and frozen at -85°C for later analysis.

2.1.7 Biochemical Markers

Serum c-terminal telopeptides of type 1 collagen were measured using a "Ratlaps" ELISA kit (Osteometer Biotech A/S, Herlev, Denmark).

2.1.8 Biomechanics

The right femurs were scraped clean of flesh and incubated at 23°C for 1 hour prior to testing so as to be at room temperature during the test. A three point bending test was used (Shimadzu Ezi-test, Kyoto, Japan), with a support span of 12 mm and a test speed of 50mm/min.

2.1.9 Bone Ash Content

The left femurs were scraped clean of flesh, oven dried for 12 hours at 105°C, measured using callipers and weighed. Dried bones were ashed at 660°C for 12 hours then re-weighed.

2.1.10 Urinary Calcium, Phosphate and Creatinine Concentration

Urinary calcium concentration was determined colorimetrically using a COBAS FARA II system and the chromagen o-cresolphthalein complexone (0.3mMol/L), 8-hydroxyquinoline (13.8mMol/L) and hydrochloric acid (122nMol/L) as supplied in a Roche Diagnostics kit (Roche Cat. No. 1 489 216).

Urinary phosphate concentration was determined photometrically at 340nm using a COBAS FARA II system and the phosphate reagent ammonium molybdate (1.0mMol/L), sulphuric acid (0.36mMol/L) and sodium chloride (43mMol/L) as supplied in a Roche Diagnostics kit (Roche Cat. No. 1 489 348).

Urinary creatinine concentration was determined colorimetrically using a COBAS FARA II system and the reagent picric acid (25mMol/L). Kit supplied by Roche Diagnostics (Cat. No. 1 489 291. Creatinine Jaffé).

2.1.11 Plasma 25-Hydroxyvitamin D_3 Concentration

Plasma 25-hydroxyvitamin D_3 concentration was determined using a ^{125}I RIA kit supplied by DiaSorin, Stillwater, Minnesota, USA.

2.1.12 Plasma Lipid Analysis

The method used to extract and analyse plasma lipids was that described by Caruso, U. et al (1991) and is based on the AOCS official method Ce 1b-89 (1992) 'Fatty acid composition by GLC-marine oils'. Basically, plasma lipids were extracted by mixing 0.8ml plasma with 1ml of internal standard solution (100µg/ml C23:0 methyl ester in 1:1 chloroform:methanol). A further 8ml of 1:1 chloroform:methanol was added and the mixture then shaken for 10 minutes on a flat bed shaker. Sample was then centrifuged and the chloroform/methanol/water phase collected. 2.4ml water and 4ml chloroform were added and the sample was re-centrifuged. The upper aqueous layer was then collected. Chloroform was evaporated and the resultant residue methylated by adding 1.5ml of 0.5M NaOH in methanol and heating at 75-80°C for 10 minutes. Sample was then cooled and 2ml of 14% BF3 methanol complex added. Sample was heated at 75-80°C for a further 30 minutes to enable the reaction to take place. After methylation, 5ml of saturated NaCl was

added and the sample extracted with 2 x 1ml of iso octane. 100µl of the iso octane extract was analysed by GLC-FID using a Aglient 6890 GC with auto sampler. Column restek rtx2330 (90% biscyanopropyl, 10% phenylcyanopropyl), 105 metre x 0.25mm id 0.1µm film thickness. The injection volume was 0.1µl splitflow EPC 1.4mL/min (20cm/s liner flow rate), constant flow mode. Head pressure at start of run was 43.33 psi. The thermal program used was 110°C hold for 2 mins, 4.00°C/min to 250°C then hold for 10 mins. Total run time was 47mins. Detector temperature was 280°C, hydrogen flow 40mL/min, air 450.0mL/min and nitrogen make-up flow 45mL/min. Injector temperature was 250°C and split was 100:1. Standard for retention time identification sigma 189-19 (37 fatty acid methyl esters from C4:0 to C24:0). Results were corrected for FID response using theoretical FID response factors. C23:0 was used as an internal standard.

2.1.13 Statistical Analyses

Results were analysed using Minitab version 13. A p-value of less than 0.05 was considered to be significant. Groups of animals were compared using one-way analysis of variance, followed by post-hoc testing (Tukey). Statistical correlations between different outcome measures were analysed using Pearson Correlation tests. Values and graphs are expressed and shown as mean ± standard error of the mean (SEM).

2.2 The Ovariectomised Rat as a Model for Postmenopausal Osteoporosis

The ovariectomised rat is commonly used as a model for postmenopausal osteoporosis. The 1994 FDA Guidelines for Preclinical and Clinical Evaluation of Agents Used in the Treatment or Prevention of Postmenopausal Osteoporosis recommend that agents be tested in the ovariectomised rat and a second, non-rodent model (Thompson *et al*, 1995).

Various studies have reported similarities between ovariectomy-induced bone loss in rats and postmenopausal bone loss in humans (Gnudi *et al*, 1993; Giardino *et al*, 1993). Shared characteristics include:

- increased bone turnover rate with rate of resorption exceeding that of formation
- initial rapid bone loss followed by a much slower rate of bone loss
- greater loss of trabecular compared to cortical bone
- decreased intestinal calcium absorption
- protective effect of obesity
- similar responses to treatment with estrogen, bisphosphonates, parathyroid hormone, calcitonin and tamoxifen
- protective effect of exercise (Kalu, 1991).

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 (Kalu, 1991). As a consequence, the ovariectomised rat principally exhibits the effects of estrogen deficiency whereas postmenopausal women are likely to exhibit the effects of estrogen deficiency as well as age-related metabolic changes depending on their age at menopause.

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. 12 months after ovariectomy indices of trabecular bone turnover in rats return to those of sham controls (Thompson *et al,* 1995).

Rat epiphyses remain open well passed the attainment of maturity and only close in female rats at age 6-8months. The mechanical loading pattern on rat bones differs from that of humans. Bone turnover rates at various skeletal sites differ from those seen in humans and rat bones are more metabolically active than human bones. This results in rat bones being more sensitive to factors that influence bone metabolic rate than human bones (Brown, 2001).

Surgical menopause results in lower vertebral and femoral neck densities than natural menopause in age-matched women (Hadkidakis *et al,* 1999; Yildiz *et al,* 1996). It is

likely therefore that ovariectomised rats exhibit exaggerated bone mineral losses compared to those occurring as a result of estrogen deficiency in postmenopausal women.

2.3 Dual Energy X-Ray Absorptiometry

Dual Energy X-Ray Absorptiometry (DEXA) involves passing x-ray beams of two different energies (usually 40kV and 70kV) through an object in order to determine the amount of soft tissue and bone matter. It is based on the principle that the attenuation of an x-ray beam is related to the thickness, density and chemical composition of the object through which it passes. Prediction equations are used to convert attenuation coefficients into body composition estimates (Ellis, 2000).

DEXA is currently considered the "gold standard" technique for measuring bone mineral density in humans (Brown, 2001). Various studies have shown that it has sufficient accuracy and precision (<2%) to allow its use in small animal studies (Ladizesky *et al,* 1994; Griffin *et al,* 1993; Ammann *et al,* 1992). A strong correlation between DEXA estimates of bone mineral content and ashed calcium measurements in rats has also been reported however DEXA estimates of bone mineral content are usually higher than measurements obtained by bone ashing (Brown, 2001).

One drawback to the use of DEXA for obtaining bone density measures is that DEXA measures area rather than true volumetric bone density. This means that a large bone will tend to be reported as having a higher bone density than a small bone (Jiang *et al,* 2000). In a longitudinal study such as this one where the density of like bones are compared, this should not be an issue. As body weight is usually highly correlated with bone size, correcting DEXA measurements for differences in body weight is a means of correcting for this.

2.4 Biochemical Markers of Bone Metabolism

The concentration of certain molecules and fragments of molecules in blood or urine can be used as an indication of the rate of bone resorption or formation. Bone formation markers are molecules produced either directly or indirectly by active osteoblasts. Levels of these markers can be measured in either the serum or plasma. Most bone resorption markers are degradation products of bone collagen and can be measured in either the urine or blood (Delmas *et al,* 2000). Assuming the processes of resorption and formation are coupled, information regarding the level of either a marker of bone formation or resorption can be extrapolated to provide an indication of the rate of bone turnover.

Biochemical markers are a sensitive indicator of changes in bone turnover rate. They provide insight into events at the molecular level of bone metabolism and therefore can be used to detect alterations in bone turnover before significant changes in bone density are observed. However, many commonly used markers are expressed in tissue other than just bone. Some drugs and diseases may interfere with marker metabolism and in some cases the available assays used to detect markers lack the required level of specificity. The limitations pertaining to the use of each biochemical marker need to be considered when interpreting assay results.

2.4.1 Type-1 Collagen c-Terminal Telopetides

Osteoclastic degradation of type 1 collagen fibres from bone matrix results in the release of specific N-terminal and C-terminal fragments of the collagen molecule into circulation. Although type-1 collagen is present in several tissues in the body it is only degraded by osteoclasts in bone, therefore measurement of serum or urine levels of either the N- or C-terminal fragments serve as a specific marker of bone resorption. In this instance levels of the C-terminal fragment (CTX) were measured in the serum. Some anomalies have been reported with the CTX serum assay however which need to be considered when analysing results. The CTX serum assay is not increased in Paget's disease, a bone disease associated with increased resorption and is increased in osteoporotic patients receiving anabolic steroids, a treatment believed to reduce bone resorption. Therefore although serum CTX

level theoretically serves as a specific marker of osteoclastic activity, in some pathological conditions it may reflect total bone turnover rather than just resorption. Serum CTX level only reflects one aspect of osteoclastic action ie degradation of type 1 collagen fibres in the organic matrix. In some pathological conditions type 1 collagen fibre synthesis may be reduced which leads to decreased degradation. In other instances, an excess of collagen or unmineralised bone matrix may exist therefore degradation appears elevated.

2.5 Biomechanical Testing

The purpose of biomechanical testing is to measure the mechanical properties of bones. Many different types of biomechanical tests exist including compression, tension, torsion, bending and fatigue tests. A 3-point bending test was utilised in this instance as this test is well suited to measuring mechanical properties of small bones such as rat femurs. The procedure involves suspending dissected rat femurs horizontally between two supports. Downwards force is gradually applied halfway along the length of the bone and the degree of bone bending and the ultimate bone breaking point is measured.

There are three stages through which bone passes during the bending test. These are known as the elastic phase, plastic phase and ultimate bone breaking point. The elastic phase occurs when low levels of force are applied to bone. The bone bends, however, as soon as the force is removed the bone reverts to its original shape – bone deformation is proportional to the force applied. Organic bone matter particularly type-1 collagen, is mainly responsible for the elastic properties of bone. As the force applied to bone increases, the bone enters the plastic phase. During this phase the applied force is still insufficient to cause bone breaking but it is sufficient to cause structural damage within the bone. This is evident by the bone failing to revert to its original shape once force is removed. Here bone deformation is disproportionate to applied force. Bone plasticity is a function of both the organic and mineral composition of the bone. As the force applied to the bone is increased further the bone eventually breaks. Bone breaking point is largely a reflection of the mineral content of the bone. During biomechanical testing, these three phases of bone breaking are measured and plotted on a Load-Deformation Curve such as shown in Figure 11.

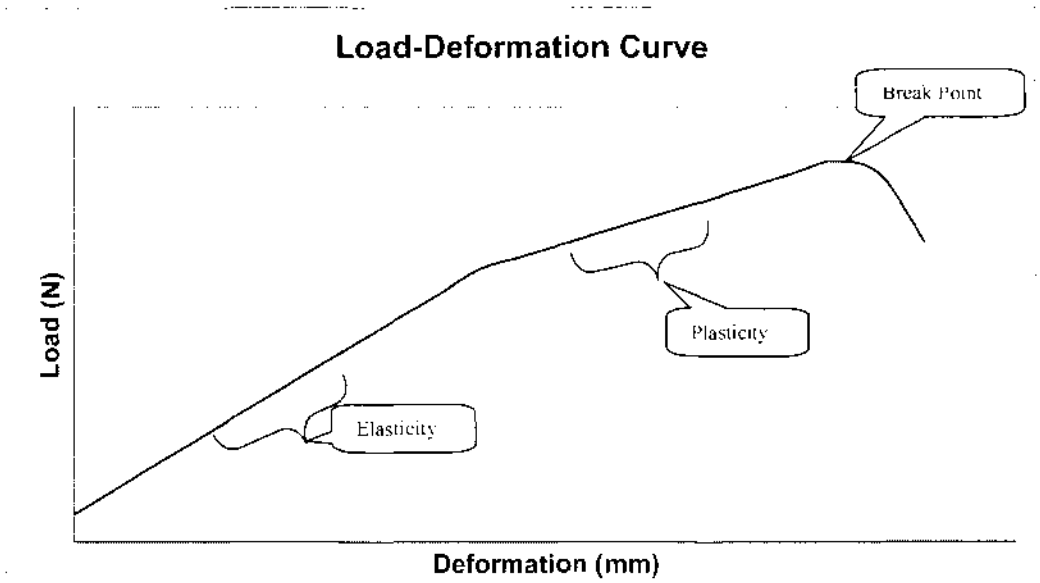


Fig 11 Load Deformation Curve Plot of bone deformation (bending) against force applied (load) showing the three phases of bone breaking - elasticity, plasticity and ultimate break point.

2.5.1 Definitions of Parameters Measured

Maximum Load (N) – the maximum force that bone can withstand without breaking.

Maximum Stroke (mm) – the maximum amount of bone deformation which occurs prior to breaking.

Break Load (N) – the force applied to bone which causes bone to break.

Break Stress (N/mm^2) – Break load expressed per area of bone.

Break Stroke (mm) – the amount of bone deformation at break point.

Break Strain (%) – the fractional change in the dimensions of the bone (degree of bending).

Elastic (N/mm^2) - a measure of the intrinsic stiffness of the bone. The slope of the linear portion of the Load-Deformation curve divided by bone area.

Energy (j) – the total amount of energy bone must absorb in order to cause a break.

SUMMARY

The ovariectomised rat is a specific model for the effects of estrogen deficiency in postmenopausal osteoporosis. It is an effective model for study durations of less than 12 months when rats are ovariectomised after the age of 6-8 months. However the effect of abrupt estrogen removal due to ovariectomy, the naturally high metabolic rate of rat bones and differences in loading patterns and regional skeletal turnover rates may result in the rat being more sensitive to factors which manipulate bone metabolic rate than humans.

DEXA is a well-established technique for measuring bone mineral content in both small animals and humans. It has a high level of precision and accuracy and is able to be used for *in vivo* measurements making it an ideal technique for measuring longitudinal changes in bone mineral density.

The use of biochemical markers as indicators of bone turnover is a relatively new technique. As changes in bone turnover due to exposure to a particular stimulus are evident earlier at the molecular level rather than at the tissue level, biochemical markers are a more sensitive measure of bone turnover than DEXA measurements. Levels of type-1 collagen crosslinks in the serum are a reflection of the level of osteoclastic activity assuming type 1 collagen metabolism within bone is normal.

DEXA and bone ash measurements provide information regarding the composition of bones. Biochemical markers provide indications as to the relative rate of bone turnover. Biomechanical testing is a method of testing the mechanical properties of bone and as such is a means of determining the actual functional significance of compositional changes or changes in bone metabolism.

CHAPTER 3

RESULTS

3.1 Diet Analysis

Protein, fat and calcium concentrations in the study diets were verified by analysis. Fatty acid analysis was carried out on both the corn oil and EPA supplement and compositions of each are as listed in Table 3.

Table 3 Percentage composition of fatty aids in the corn oil and EPA supplement used in study diets

C16	12.2	<0.1
C16:1	0.1	<0.1
C17	0.1	<0.1
C18	2.2	<0.1
C18:1 Oleic	32.5	<0.1
C18:1 cis 11	0.6	<0.1
C18:2	49.3	<0.1
C20/gamma C18:3	0.6	<0.1
C18:3	0.8	<0.1
C20:1	0.2	<0.1
Unknowns	1.4	<0.1
C20:5	<0.1	99.0

3.2 Uterus Weight

Uterus weights were recorded at week 10 as a means of confirming effectiveness of ovariectomy. Uterus weight of one rat originally assigned to the OVX control group revealed ovariectomy had not been successful and as a result this rat was re-assigned to the sham group. There was no significant difference in uterus weight between the high dose

EPA, low dose EPA and OVX groups (p=1.0). Sham rats had significantly higher uterus weights than all other groups (p<0.0001 for all, refer Table 4).

3.3 Rat Weight

There was no significant difference between groups in terms of rat weight at week 0 (322.1 ± 1g). As shown in Table 4 sham rats were significantly lighter than ovariectomised rats (p=0.0001) at week 10. There was no significant difference in rat weight between the three ovariectomised rat groups at week 10 (p=1.0).

Table 4 Final uterus and total body weights following ovariectomy or sham operation and 9 weeks of feeding a corn oil or EPA supplemented diet.

Uterus Weight (g)	0.8581^{a}	0.2269^{b}	0.2343^{b}	0.2327^{b}
SE	0.03821	0.0424	0.0409	0.03946
Rat Weight (g)	335.49^{a}	376.98^{b}	375.45^{b}	375.66^{b}
SE	6.34	5.32	5.19	8.06

Reported means are Least Squares Mean Values. Different superscripts (a,b,c) denote significant differences between groups at p<0.05.

3.4 Urinary Calcium and Phosphate Excretion

Possible trends were evident for urinary calcium excretion and the ratio of calcium:phosphate in the urine. The OVX group had higher values for both measures compared to the sham group (p=0.1836 and p=0.1283 respectively). There was no significant difference between groups in urinary phosphate excretion. Results are shown in Table 5.

Table 5 Urinary calcium and phosphate concentrations following ovariectomy or sham operation and 9 weeks of feeding a corn oil or EPA supplemented diet.

Calcium excretion (mMol Ca/mMol creatinine)	Mean	0.3511[a]	0.5521[a]	0.4763[a]	0.4973[a]
	SE	0.0715	0.0668	0.0668	0.0705
Phosphate excretion (mMol PO4/mMol creatinine)	Mean	5.551[a]	5.284[a]	5.723[a]	5.148[a]
	SE	0.5214	0.4946	0.4946	0.5214
Ratio of Ca:PO4 in urine (corrected for creatinine)	Mean	0.0668[a]	0.1132[a]	0.0861[a]	0.1012[a]
	SE	0.0149	0.0142	0.0142	0.0149

Reported means are Least Squares Mean Values. Different superscripts (a,b,c) denote significant differences between groups at $p<0.05$.

3.5 Serum C-Telopeptides of Type 1 Collagen (CTX)

There were no significant differences between groups for serum CTX concentrations at baseline ($p=1.0$ for all, data not shown). Rat weight was not significantly correlated with serum CTX concentration (data not shown) therefore CTX values were analysed assuming no confounders.

At week 10, the high dose EPA group had significantly higher serum CTX concentrations than the sham group ($p=0.0032$). Although not statistically significant, a clear trend was evident between the high dose EPA and OVX groups ($p=0.14$). The low dose EPA group had higher serum CTX concentrations than the sham group however this also failed to reach significance ($p=0.1052$). There were no significant differences between any of the other groups (refer Table 6).

Table 6 Serum type-1 collagen c-telopeptide concentrations following ovariectomy or sham operation and 9 weeks of feeding a corn oil or EPA supplemented diet.

Mean Concentration (ng/mL)	4.485[a]	6.744[a,b]	7.855[a,b]	9.901[b]
SE	0.984	0.984	1.037	1.037

Different superscripts (a,b,c) denote significant differences between groups at $p<0.05$.

3.6 Bone Density

3.6.1 Lumbar Spine

Lumbar spine bone mineral content and density comparisons were made based on data from *in vivo* scans at baseline as well as week 10. Bone mineral content and density data were corrected for rat weight. At baseline there was no significant difference between the study groups for either bone mineral content (BMC) or bone mineral density (BMD) (data not shown).

At week 10, rat weight was a significant predictor of lumbar spine (LS) BMC ($p=0.03$) but not LS BMD ($p=0.5$) (data not shown). Study group was a highly significant predictor of both LS BMC ($p<0.001$) and LS BMD ($p<0.001$) (data not shown). After correction for body weight, there was a highly significant difference in LS BMC between the sham group and OVX ($p=0.003$), low dose EPA ($p=0.01$) and high dose EPA ($p=<0.0001$) groups with the sham group having considerably higher BMC than the other groups (data not shown). As shown in Table 7, there was no significant difference between the OVX group and low dose EPA group ($p=0.9$). A possible trend was evident between the low dose and high dose EPA groups ($p=0.17$) with BMC in the high dose group being 9.0% lower than that in the low dose group.

Highly significant differences were evident in LS BMD between the sham group and all other groups. These equated to decreases in LS BMD of 18.2% in the high dose group ($p=<0.001$), 10.1% in the low dose group ($p=0.004$) and 12.2% in the OVX group ($p=<0.001$). There was no significant difference in LS BMD between the OVX and low

dose EPA group (p=0.88). However a 6.8% decrease in LS BMD was evident in the high dose EPA group compared to the low dose group (p=0.04). Results are shown in Table 7.

Table 7 Lumbar spine bone mineral content and density measures following ovariectomy or sham operation and 9 weeks of feeding a corn oil or EPA supplemented diet.

BMC (g)	Mean	0.5656^{a}	0.4817^{b}	0.4933^{b}	0.4524^{b}
	SE	0.01546	0.01478	0.01417	0.01425
BMD (g/cm2)	Mean	0.2547^{a}	$0.2270^{b,c}$	0.2313^{b}	0.2155^{c}
	SE	0.00463	0.00442	0.00424	0.00427

Reported means are Least Squares Mean Values. Different superscripts (a,b,c) denote significant differences between groups at $p<0.05$.

Figures 12 and 13 illustrate the change in lumbar spine BMC and BMD respectively from baseline to study completion.

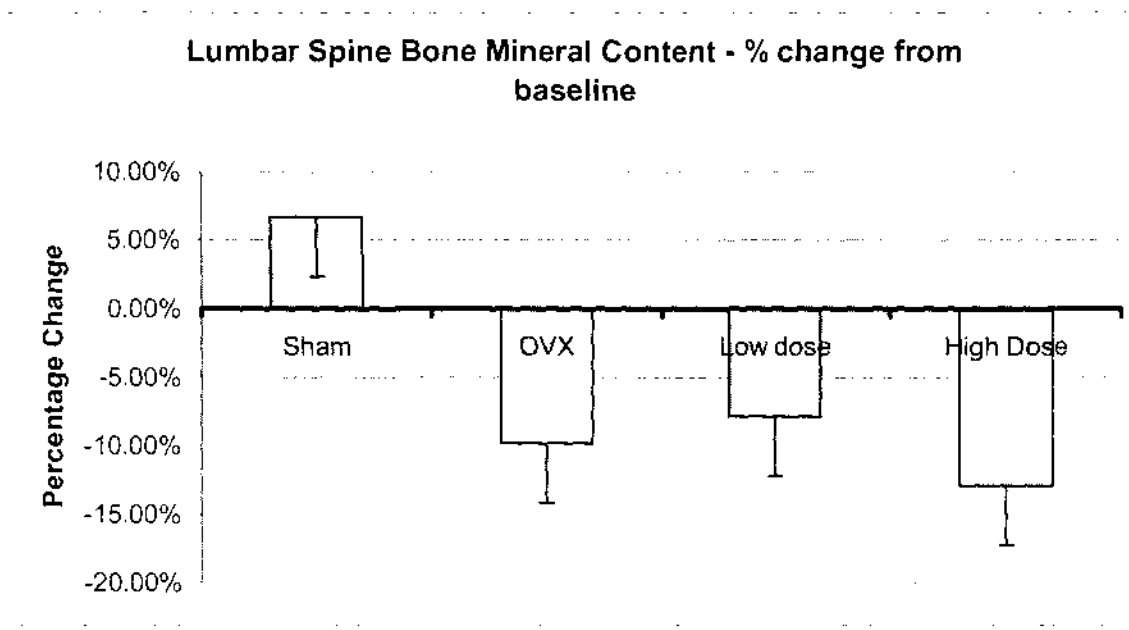


Fig 12 Lumbar Spine Bone Mineral Content - % change from baseline. Change in LS BMC as measured by DEXA following ovariectomy or sham operation and 9 weeks of feeding a corn oil or EPA supplemented diet. Values were calculated as follows: (LS BMC_{final} - LS $BMC_{baseline}$)/LS $BMC_{baseline}$.

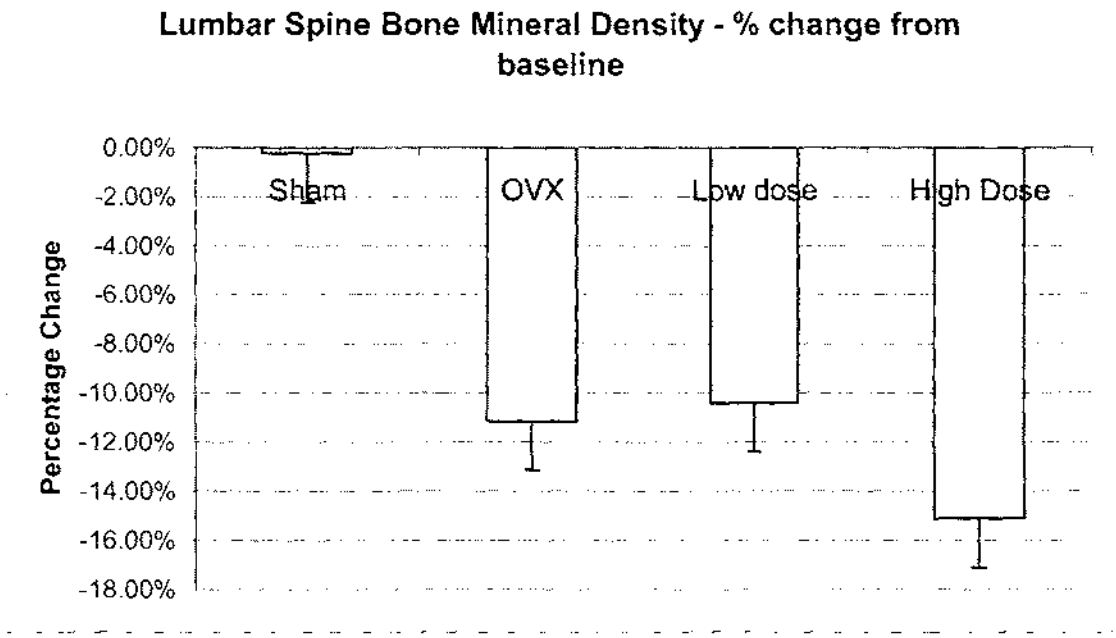


Fig 13 Lumbar Spine Bone Mineral Density - % change from baseline. Change in LS BMD as measured by DEXA following ovariectomy or sham operation and 9 weeks of feeding a corn oil or EPA supplemented diet. Values were calculated as follows: (LS BMD_{final} - LS $BMD_{baseline}$)/LS $BMD_{baseline}$.

3.6.2 Femurs

Study group was a highly significant predictor of both femur BMC and BMD ($p<0.001$). Rat weight was not a significant predictor of femur BMD ($p=0.47$) but a slight correlation was evident for femur BMC ($p=0.1$). Rat weight was treated as a confounder when BMC and BMD values were analysed. A possible source of error in obtaining *in vivo* DEXA measurements of femurs derives from the difficulty of excluding areas of tibial and pelvic bones from the analysis. For this reason DEXA measures obtained by scanning the femurs *ex vivo* were used for the final BMC and BMD comparisons however baseline DEXA scans were conducted on femurs *in vivo*. Measures of both right and left femurs were taken and the results were pooled.

There was no significant difference between the groups at baseline for either *in vivo* femur BMC or BMD.

Decreased *ex vivo* femur BMC was evident in all three groups compared to sham rats at week 10 but this difference only reached significance for the low dose and high dose EPA groups ($p=0.01$ and $p<0.001$ respectively). The high dose EPA group had the lowest femur BMC of all groups. The OVX group exhibited a 6.0% decrease in femur BMC compared to shams, the low dose group had a 9.1% decrease and the high dose group a 14.9% decrease. Femur BMC in the high dose group was also significantly lower than the OVX group ($p=0.008$) and a possible trend was evident between the high and low dose groups ($p=0.1$).

The sham group had significantly higher femur BMD than all other groups ($p<0.001$ for all) whereas the high dose EPA group had significantly lower femur BMD than all other groups (low dose $p=0.0047$, OVX $p=0.0096$). There was no significant difference between the OVX and low dose EPA groups ($p=0.995$). Table 8 depicts the final *ex vivo* femur BMC and BMD results obtained.

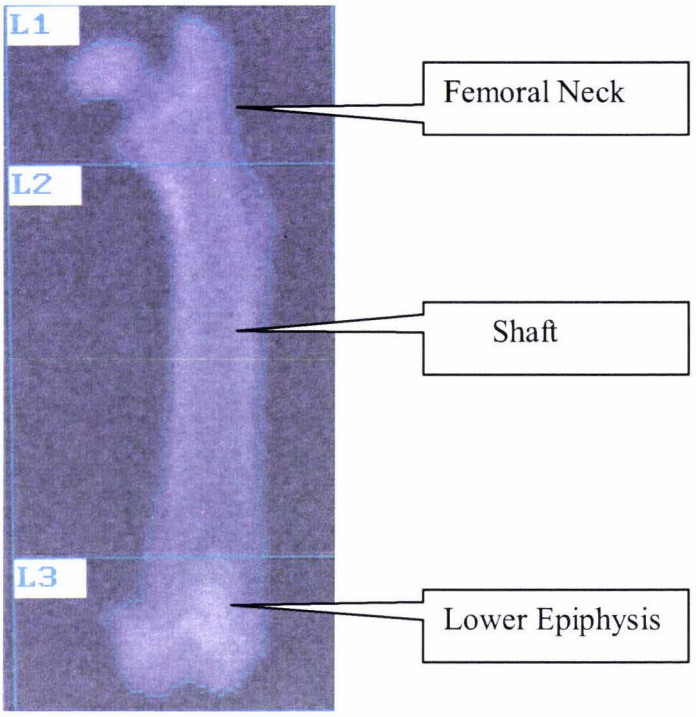
Table 8 Ex vivo femur bone mineral content and density measures following ovariectomy or sham operation and 9 weeks of feeding a corn oil or EPA supplemented diet.

BMC (g)	Mean	0.5563^{a}	$0.5250^{a,b}$	$0.5099^{b,c}$	0.4840^{c}
	SE	0.0104	0.0094	0.0091	0.0090
BMD	Mean	0.2851^{a}	0.2567^{b}	0.2575^{b}	0.2435^{c}
(g/cm2)	SE	0.0034	0.0031	0.0030	0.0029

Reported means are Least Squares Mean Values. Different superscripts (a,b,c) denote significant differences between groups in the same row at $p<0.05$.

BMC and BMD measurements were also obtained by region for *ex vivo* femurs. These regions were assigned as shown in Figure 14 and are subsequently referred to as the femoral neck, shaft and lower epiphysis although some non-epiphyseal bone matter will be present in the epiphyseal region.

Fig 14 Designation of Femur Regions for analysis of ex vivo Bone Mineral Content and Density.



BMC of the lower epiphysis was significantly reduced in all groups compared to sham ($p<0.001$ for all). A decrease in BMC was also evident for the lower epiphysis in the high dose EPA group compared to the low dose EPA and OVX groups however these differences failed to reach significance ($p=0.13$ and $p=0.09$ respectively). There was no significant difference between the low dose EPA and OVX groups. All groups exhibited significantly reduced lower epiphysis BMD compared to the sham group ($p<0.001$ for all). BMD values for the lower epiphysis were also reduced in the high dose EPA group compared to all other groups. The difference was significant for the high dose EPA vs sham group ($p<0.001$) as well as high dose EPA vs low dose EPA group ($p=0.03$) but the difference failed to reach significance when the high dose EPA and OVX groups were compared ($p=0.11$). There was no significant difference between the low dose EPA and OVX groups ($p=0.96$).

Between group comparisons of femur shaft BMC showed a significant reduction in BMC in the high dose EPA group compared to sham ($p=0.04$) and OVX ($p=0.0012$) groups. No significant differences were apparent between any of the other groups. Femur shaft BMD

was significantly lower in the high dose EPA group compared to sham ($p<0.001$), OVX ($p= 0.0036$) and low dose EPA ($p=0.0003$) groups. There was no significant difference between the low dose EPA and OVX groups ($p=0.9$) but both groups showed a significant reduction in femur shaft BMD compared to the sham group ($p=0.017$, low dose EPA and $p=0.004$, OVX).

There was no significant difference in femoral neck BMC between the OVX and sham groups ($p=0.08$) but significant reductions in BMC were apparent in the low dose EPA and high dose EPA groups compared to sham ($p=0.0187$ and $p=0.0009$ respectively). Significant reductions in femoral neck BMD values were apparent for all groups compared to the sham group ($p=0.0001$ high dose and low dose EPA groups and $p=0.0016$, OVX group). No significant differences between any of the other groups were evident. Regional femur BMC and BMD measurements are reported in Table 9.

Table 9 Final ex vivo bone mineral content and density by femur area following ovariectomy or sham operation and 9 weeks of feeding a corn oil or EPA supplemented diet.

Lower Epiphysis	BMC (g)	Mean	0.1368^{a}	0.1177^{b}	0.1172^{b}	0.1106^{b}
		SE	0.0025	0.0020	0.0022	0.0020
	BMD (g/cm2)	Mean	0.2960^{a}	$0.2601^{b,c}$	0.2628^{b}	0.2476^{c}
		SE	0.0046	0.0041	0.0040	0.0039
Shaft	BMC (g)	Mean	0.2700^{a}	0.2752^{a}	$0.2647^{a,b}$	0.2514^{b}
		SE	0.0051	0.0046	0.0050	0.0044
	BMD (g/cm2)	Mean	0.2615^{a}	0.2428^{b}	0.2457^{b}	0.2270^{c}
		SE	0.0037	0.0033	0.0032	0.0032
Femoral neck	BMC (g)	Mean	0.1495^{a}	$0.1321^{a,b}$	0.1281^{b}	0.1219^{b}
		SE	0.0051	0.0046	0.0044	0.0044
	BMD (g/cm2)	Mean	0.3280^{a}	0.2895^{b}	0.2820^{b}	0.2823^{b}
		SE	0.0071	0.0063	0.0062	0.0061

Reported means are Least Squares Mean Values. Different superscripts (a,b,c) denote significant differences between groups in the same row at $p<0.05$.

3.7 Bone Ash Content

Bone length was a significant predictor of dry bone weight ($p<0.0001$) (data not shown). A possible trend was evident between rat weight and dry bone weight ($p=0.088$). Both bone length and rat weight were treated as confounders when dry bone weight results were analysed. As shown in Table 10, dry bone weight was highly correlated with study group ($p<0.0001$). The high dose EPA group had significantly lower dry bone weight than the OVX ($p=0.0012$), sham ($p<0.0001$) and low dose EPA ($p=0.0217$) groups. There was no significant difference in dry bone weight between the OVX and sham group ($p=0.24$) or low dose EPA and OVX group ($p=0.74$). The low dose EPA group had significantly lower dry bone weight than the sham group ($p=0.033$).

Table 10 Dry femur weight and composition following ovariectomy or sham operation and 9 weeks of feeding a corn oil or EPA supplemented diet.

Dry Bone Length (mm)	Mean	36.44[a]	37.16[a]	36.94[a]	37.25[a]
	SE	0.2483	0.2577	0.2483	0.2399
Dry Bone Weight (g)	Mean	0.6659[a]	0.6404[a,b]	0.6289[b]	0.5958[c]
	SE	0.0095	0.0084	0.0081	0.0078
Ashed Bone Weight (g)	Mean	0.3803[a]	0.3476[a,b]	0.3464[a,b]	0.3395[b]
(Bone Mineral)	SE	0.0096	0.0088	0.0082	0.0079
Non-Mineral Bone	Mean	0.2753[a]	0.2922[a,b]	0.2797[a,b.]	0.2637[b]
Matter (g)	SE	0.0068	0.0074	0.0068	0.0066
Ratio non-mineral:	Mean	0.7320[a]	0.8487[a]	0.8076[a]	0.7784[a]
mineral bone matter*	SE	0.0321	0.0294	0.0274	0.0265

Reported means are Least Squares Mean Values. Different superscripts (a,b,c) denote significant differences between groups in the same row at $p<0.05$.

* Ratio calculation: Non-mineral Bone matter (g)/Ashed Bone Weight (g).

Ashed bone weight was highly correlated with rat weight ($p=0.005$) and study group ($p=0.026$). The high dose EPA group had significantly lower ashed bone weight after correction for body weight than the sham group ($p=0.01$) but not the OVX ($p=0.89$) or low dose EPA ($p=0.92$) groups. There was no significant difference between the low dose EPA

and OVX groups (p=1.00). The sham group had higher ashed bone weight than both the low dose EPA and OVX groups however these differences just failed to reach significance (p=0.06 and p=0.09 respectively). Results are illustrated in Figure 15.

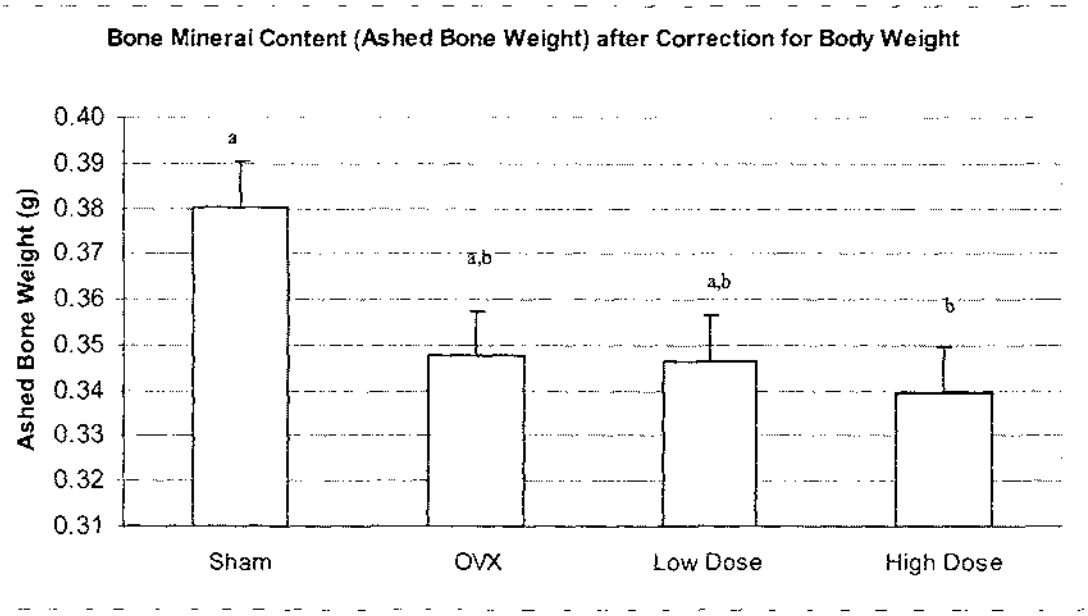


Fig 15 Bone Mineral Content (Ashed Bone Weight) after Correction for Body Weight.
Least Square Mean values for bone mineral content by study group following ovariectomy or sham operation and 9 weeks of feeding a corn oil or EPA supplemented diet as measured by ashing left femurs at 660°C overnight. Different letters (a,b,c) denote significant differences between groups at $p<0.05$.

The amount of non-mineral bone matter was calculated by subtracting ashed bone weight from dry bone weight. Rat body weight was not a significant predictor of non-mineral bone matter (p=0.400) however a significant correlation with study group was found (p=0.047) (data not shown). The high dose EPA group had significantly lower non-mineral bone matter than the OVX group (p=0.02) but there was no significant difference in amount of non-mineral bone matter between any of the other groups.

The ratio of non-mineral:mineral bone matter was calculated by dividing the weight of non mineral bone matter by ashed bone weight. A possible trend was evident between rat body weight and non-mineral:mineral bone matter (p=0.14) therefore rat weight was treated as a confounder. Study group was also correlated with non-mineral:mineral bone matter

however this just failed to reach significance (p=0.078). The non-mineral:mineral bone matter ratio was higher in the OVX group compared to the sham group however this just failed to reach significance (p=0.06) (refer Figure 16).

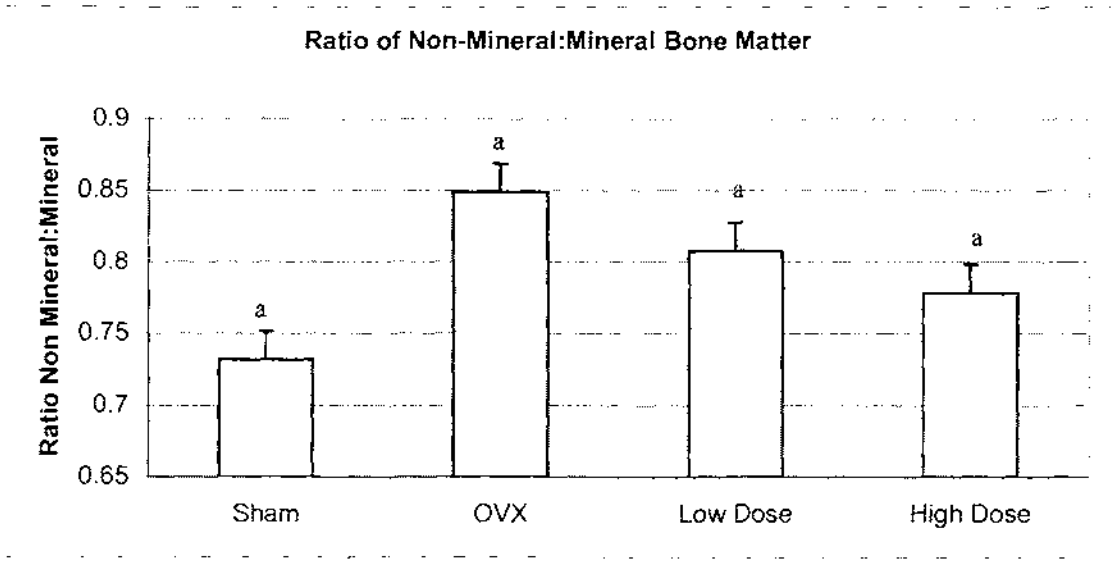


Fig 16 Ratio of Non-Mineral:Mineral Bone Matter. Ratio of non-mineral:mineral bone matter following ovariectomy or sham operation and 9 weeks of feeding a corn oil or EPA supplemented diet. Values were calculated by subtracting ashed bone weight from dry bone weight to obtain an estimate of non-mineral bone mass. Weight of non-mineral bone matter was then divided by the ashed bone weight to obtain the non mineral:mineral ratio. Different letters (a,b,c) denote significant differences between groups at $p<0.05$.

3.8 Bone Breaking Strength

There was no significant correlation between rat weight, femur wet weight or femur length and any of the parameters measured during biomechanical testing of the femurs. Therefore data were analysed assuming there were no confounders.

As shown in Table 11, there was no significant difference between groups for maximum stroke or break strain. There was possibly a slight group effect for maximum load, break load, break stroke and energy however a significant difference between groups was not

evident for any of these parameters (p=0.091, p=0.105, p=0.139, p=0.181 respectively). A significant group effect was evident for break stress (p<0.001) and elasticity (p=0.05).

Table 11 Results of biomechanical tests on right femurs following ovariectomy or sham operation and 9 weeks of feeding a corn oil or EPA supplemented diet.

Max Load (N)	Mean	240.51[a]	218.57[a]	230.23[a]	216.82[a]
	SE	8.30	6.39	7.92	7.01
Max Stroke (mm)	Mean	1.702[a]	1.635[a]	1.617[a]	1.615[a]
	SE	0.031	0.047	0.034	0.034
Break Load (N)	Mean	237.10[a]	213.38[a]	224.67[a]	211.70[a]
	SE	10.70	5.80	7.28	7.59
Break Stress (N/mm2)	Mean	119.44[a]	90.97[b]	106.48[a,b]	100.08[b]
	SE	5.20	4.17	3.67	4.33
Break Stroke (mm)	Mean	1.713[a]	1.610[a]	1.616[a]	1.620[a]
	SE	0.035	0.037	0.036	0.031
Break Strain (%)	Mean	14.91[a]	15.00[a]	14.58[a]	14.67[a]
	SE	0.579	0.342	0.504	0.492
Elastic (N/mm2)	Mean	885.5[a]	686.9[b]	811.2[a,b]	811.3[a,b]
	SE	63.5	35.3	49.2	46.9
Energy (J)	Mean	0.207[a]	0.182[a]	0.190[a]	0.186[a]
	SE	0.010	0.008	0.009	0.008

Different superscripts (a,b,c) denote significant differences between groups in the same row at p<0.05.

There was a highly significant difference between the break stress for the OVX group compared to that of the sham group (p=0.0002) with the OVX group having a much lower break stress (90.97N/mm2) compared to the sham group (119.44N/mm2). There was no significant difference between the low dose EPA group and the sham group (p=0.18) whereas the difference between the OVX and low dose EPA groups was approaching significance (p=0.07).

There was a significant difference between the OVX and sham groups for bone elasticity (p=0.019) with the sham group having stiffer bones than OVX however there was no significant difference between any of the other groups.

3.9 Plasma Lipid Content

Due to budget constraints, lipid analysis was conducted on plasma samples from the OVX (n=10), low dose EPA (n=10) and high dose EPA (n=10) groups only. Results are summarised in Table 12. There was no significant difference between the low dose EPA and OVX groups as to total plasma lipid content ($p=0.8570$). The high dose EPA group had substantially lower total plasma lipid levels than both the low dose EPA ($p=0.0185$) and OVX ($p=0.0051$) groups. There was no significant difference between groups as to the percentage of saturated, monounsaturated and polyunsaturated fatty acids in plasma membranes.

Both EPA supplemented groups had a higher percentage of linoleic acid in plasma membranes than the ovariectomised controls. This difference was only significant however for the low dose EPA group vs OVX ($p=0.0338$). There was no significant difference between groups as to the percentage of α-linolenic acid although both EPA supplemented groups had higher levels than the non-supplemented controls. The high does EPA group had the highest percentage of n-3 fatty acids and lowest percentage of n-6 fatty acids in plasma. The OVX group had the lowest percentage of n-3 and highest percentage of n-6 fatty acids whereas the low dose EPA group had values in between those of the OVX and high dose EPA groups. All differences between groups were highly significant ($p<0.0001$). Highly significant differences between all three groups were also evident for arachidonic acid concentration with the highest concentration being found in the ovariectomised group and lowest concentration in the high dose EPA group ($p<0.005$). In contrast, the high dose EPA group had the highest plasma EPA and Docosapentaenoic Acid (DPA) concentration and the OVX group the lowest. Again the differences between all groups were significant ($p\leq0.01$). The low dose EPA group had the highest concentration of DHA in plasma. The concentration was significantly greater than that of the high dose EPA group ($p=0.0006$) but not significantly different from that of the OVX group ($p=0.2319$). The high dose EPA group had significantly lower plasma DHA concentration than both the low dose EPA ($p=0.0006$) and OVX groups ($p=0.0394$).

concentrations of 25(OH) vitamin D_3 compared to both the OVX ($p=0.0006$) and sham groups ($p<0.0001$). There was no significant difference in 25(OH) vitamin D_3 concentration between the high dose and low dose EPA groups ($p=0.5573$). The high dose EPA group also had significantly higher serum 25(OH) vitamin D_3 concentration than the OVX ($p=0.0224$) and sham ($p=0.0004$) groups. There was no significant difference between the OVX and sham groups ($p=0.4697$).

Table 13 Serum 25(OH) Vitamin D_3 concentration following ovariectomy or sham operation and 9 weeks of feeding a corn oil or EPA supplemented diet.

Mean	9.967^{a}	12.189^{a}	18.536^{b}	16.680^{b}
SE	1.074	1.074	0.971	1.019

Reported means are Least Squares Mean Values. Different superscripts (a,b,c) denote significant differences between groups in the same row at $p<0.05$.

3.11 Pearson Correlations

The relationship between the various outcomes measured was analysed using a Pearson Correlation test in order to verify consistency across the range of results obtained. Results for the 30 rats (10 OVX, 10 low dose EPA, 10 high dose EPA) for which plasma fatty acid composition was determined were used for the Pearson Correlation test. Trends would therefore be valid for an ovariectomised animal only.

As shown in Table 14, serum 25(OH) vitamin D_3 concentration was significantly, negatively correlated with femur BMD ($p=0.024$). Femur BMD was also significantly correlated with bone ash ($p<0.001$) and organic:ash ratio ($p=0.013$). Serum CTX concentration was not significantly correlated with any other measure. Urinary calcium and phosphate ratio excretion were significantly correlated ($p=0.01$).

Table 14 Pearson Correlations between measurements of bone composition, strength, markers of bone metabolism and calcium and phosphate balance following ovariectomy or sham operation and 9 weeks of feeding a corn oil or EPA supplemented diet.

		25OH Vit D	BMD	Bone Ash	Organic Matter	Organic :Ash	CTX	Urinary Ca	Urinary PO4
Femur BMD	*Pearson Corr.*	-0.356							
	p value	0.024							
Bone Ash	*Pearson Corr.*	-0.092	0.540						
	p value	0.577	0.000						
Organic Matter	*Pearson Corr.*	-0.134	-0.002	0.126					
	p value	0.416	0.988	0.392					
Organic: Ash	*Pearson Corr.*	-0.041	-0.355	-0.618	0.692				
	p value	0.806	0.013	0.000	0.000				
Serum CTX	*Pearson Corr.*	0.146	-0.235	0.042	0.111	0.063			
	p value	0.476	0.196	0.818	0.546	0.734			
Ca excretion	*Pearson Corr.*	-0.049	-0.074	0.181	0.083	-0.067	0.222		
	p value	0.797	0.679	0.305	0.642	0.705	0.275		
PO4 excretion	*Pearson Corr.*	-0.082	0.151	-0.026	-0.138	-0.072	0.182	0.437	
	p value	0.668	0.394	0.884	0.435	0.687	0.374	0.010	
Urinary Ca:PO4	*Pearson Corr.*	0.015	-0.134	0.133	0.172	0.017	0.049	0.423	-0.475
	p value	0.938	0.449	0.454	0.332	0.923	0.813	0.013	0.005

Key: Red Font Significant correlation between measures at $p<0.05$.
Blue Font Indicates a possible trend ($p=0.1$)
Black Font $p>0.1$

Pearson correlation tests were also performed on the plasma lipid composition data for the 30 rats for which this measurement was made in order to determine if statistically significant correlations existed between the relative concentrations of the various EFAs. As shown in Table 15, plasma concentration of all EFAs except DHA were significantly correlated with each other. An inverse correlation between the %n-3 and %n-6 EFAs in plasma occurred ($p<0.001$).

Table 15 Pearson Correlations between EFAs in rat plasma following ovariectomy or sham operation and 9 weeks of feeding a corn oil or EPA supplemented diet.

		EPA	DPA	DHA	AA	GLA	%n-3
DPA	*Pearson Corr.*	0.936					
	p value	0.000					
DHA	*Pearson Corr.*	-0.554	-0.428				
	p value	0.002	0.023				
AA	*Pearson Corr.*	-0.930	-0.927	0.480			
	p value	0.000	0.000	0.010			
GLA	*Pearson Corr.*	-0.827	-0.862	0.155	0.798		
	p value	0.000	0.000	0.430	0.000		
% n-3	*Pearson Corr.*	0.998	0.952	-0.506	-0.937	-0.851	
	p value	0.000	0.000	0.006	0.000	0.000	
% n-6	*Pearson Corr.*	-0.956	-0.935	0.485	0.993	0.811	-0.961
	p value	0.000	0.000	0.009	0.000	0.000	0.000

Key: Red Font Significant correlation between measures at $p<0.05$.

Blue Font Indicates a possible trend ($p=0.1$)

Black Font $p>0.1$

Plasma lipid composition for the 30 rats for which this measure was made were compared to data from other outcome measures for the same rats in order to determine if any statistically significant correlations existed between the measures. Serum concentration of 25(OH) vitamin D_3 was inversely correlated with the plasma ratio of AA:EPA, GLA:EPA and GLA:DPA ($p=0.044$, $p= 0.047$ and $p=0.031$ respectively. The amount of femur organic matter was negatively associated with the plasma concentration of n-3 EFAs ($p=0.022$) and positively associated with the plasma concentration of n-6 EFAs ($p=0.019$). The strongest positive association between amount of organic matter and any single EFA was evident with GLA ($p=0.008$). The ratio of EPA:DPA had the strongest negative correlation with the amount of organic matter ($p=0.009$). DHA was the only n-3 EFA measured which was not significantly correlated with the amount of organic matter ($p=0.610$). Serum CTX concentration was positively correlated with the unsaturation index ($p=0.035$) (refer Table 16).

Table 16 Pearson Correlations between plasma EFA composition and measurements of bone composition, strength and markers of bone metabolism following ovariectomy or sham operation and 9 weeks of feeding an EPA supplemented diet.

		25(OH) Vit D	*Femur BMD*	*Bone Ash*	*Organic Matter*	*Organic: Ash*	Serum CTX	Urinary Ca	*Urinary PO4*	*Urinary Ca:PO4*
EPA	*P Corr.*	0.148	-0.302	-0.047	-0.429	-0.321	0.265	-0.079	-0.179	0.231
	p value	0.491	0.119	0.914	0.026	0.102	0.258	0.740	0.449	0.328
DPA	*P Corr.*	0.187	-0.243	-0.046	-0.407	-0.310	0.283	-0.069	-0.182	0.358
	p value	0.382	0.213	0.819	0.035	0.115	0.227	0.771	0.443	0.121
DHA	*P Corr.*	0.302	-0.001	-0.200	0.103	0.214	0.048	0.072	0.375	-0.352
	p value	0.151	0.994	0.318	0.610	0.284	0.841	0.762	0.104	0.128
AA	*P Corr.*	-0.185	0.220	0.050	0.456	0.343	-0.116	-0.069	0.108	-0.353
	p value	0.387	0.260	0.804	0.017	0.079	0.625	0.773	0.653	0.127
GLA	*P Corr.*	-0.316	0.236	0.219	0.501	0.252	-0.326	0.087	-0.026	-0.080
	p value	0.132	0.226	0.273	0.008	0.206	0.161	0.714	0.914	0.736
EPA: DPA	*P Corr.*	0.336	-0.197	-0.052	-0.492	-0.369	0.263	-0.091	-0.035	0.051
	p value	0.108	0.314	0.797	0.009	0.059	0.263	0.703	0.883	0.832
EPA: DHA	*P Corr.*	0.090	-0.264	-0.029	-0.446	-0.345	0.254	-0.079	-0.202	0.213
	p value	0.674	0.175	0.884	0.020	0.078	0.281	0.740	0.392	0.368
AA: EPA	*P Corr.*	-0.403	-0.298	-0.028	0.283	0.248	-0.303	-0.184	-0.153	-0.147
	p value	0.056	0.131	0.892	0.162	0.222	0.207	0.451	0.533	0.550
AA: DPA	*P Corr.*	-0.414	-0.179	0.023	0.303	0.237	-0.277	-0.098	-0.113	-0.166
	p value	0.044	0.361	0.911	0.124	0.234	0.238	0.682	0.635	0.486
AA: DHA	*P Corr.*	-0.329	0.294	0.179	0.502	0.294	-0.167	-0.085	-0.053	-0.210
	p value	0.116	0.129	0.372	0.008	0.137	0.482	0.721	0.825	0.373
GLA: EPA	*P Corr.*	-0.418	-0.225	0.037	0.338	0.240	-0.347	-0.177	-0.220	-0.085
	p value	0.047	0.258	0.856	0.091	0.237	0.146	0.468	0.366	0.731
GLA: DPA	*P Corr.*	-0.440	-0.116	0.091	0.338	0.209	-0.327	-0.071	-0.166	-0.092
	p value	0.031	0.556	0.650	0.085	0.296	0.159	0.766	0.483	0.701
GLA: DHA	*P Corr.*	-0.396	0.273	0.321	0.461	0.150	-0.312	0.061	-0.170	0.055
	p value	0.055	0.160	0.103	0.016	0.456	0.181	0.797	0.475	0.819
% n-3	*P Corr.*	0.173	-0.306	-0.061	-0.438	-0.321	0.278	-0.076	-0.164	0.231
	p value	0.419	0.113	0.763	0.022	0.103	0.235	0.750	0.491	0.327
%n-6	*P Corr.*	-0.167	0.274	0.060	0.450	0.333	-0.156	-0.041	0.115	-0.318
	p value	0.434	0.158	0.767	0.019	0.089	0.510	0.865	0.630	0.172
n-3: n-6	*P Corr.*	0.115	-0.311	-0.046	-0.401	-0.300	0.278	-0.061	-0.176	0.225
	p value	0.593	0.108	0.818	0.038	0.129	0.235	0.799	0.457	0.341
Unsat. Index*	*P Corr.*	0.101	-0.344	-0.062	-0.255	-0.166	0.473	-0.390	-0.234	-0.140
	p value	0.639	0.073	0.757	0.199	0.407	0.035	0.089	0.320	0.557

Key: Red Font Significant correlation between measures at $p<0.05$.

Blue Font Indicates a possible trend ($p=0.1$)

Black Font $p>0.1$

* Unsaturation Index - calculated as percentage fatty acid of total fatty acids x number of double bonds

CHAPTER 4

DISCUSSION

SECTION 1

Observations

The aim of the present study was to test the hypothesis that EPA supplementation can increase bone density in ovariectomised animals. The observed effects of low and high dose EPA supplementation on various aspects of bone turnover as measured in the current study are discussed and interpreted in order to ascertain the validity of this hypothesis.

4.1 Urinary Calcium and Phosphate Excretion

4.1.1 Effect of Ovariectomy

Urinary calcium and phosphate excretion is largely governed by dietary intake of both ions as well as the activity of vitamin D (Lemann, 1993). In the current study, dietary calcium, phosphate and vitamin D intakes were the same in both ovariectomised and sham groups however vitamin D activity may have differed as a consequence of ovariectomy. Estrogen deficiency results in decreased responsiveness of cells to $1,25(OH)_2$ vitamin D_3 (Leonard *et al.* 2001; Liel *et al*, 1999) and this may partially explain the decreased intestinal calcium absorption and decreased renal calcium reabsorption usually evident in cases of estrogen deficiency (Leonard *et al.* 2001). $1,25(OH)_2$ vitamin D_3 acts to increase active calcium and phosphate absorption in the intestine and inhibit renal reabsorption of both ions resulting in increased urinary calcium and phosphate concentrations (Norman & Henry, 1993). In the current study, ovariectomy may have increased urinary calcium excretion however results were not statistically significant. Urinary phosphate excretion was similar although slightly higher in the sham versus ovariectomised control group. Although no significant difference was apparent in the current study, reduced urinary

phospate excretion is consistent with the reported effects of estrogen deficiency (Adami *et al,* 1992). The method used in the current study to determine urinary calcium and phosphate concentrations is best suited for detecting relatively large differences in urine composition and therefore may have lacked the necessary sensitivity to detect any change that had occurred during the study period. The use of 24 hour urine samples, a more sensitive measurement method, may have yielded a more conclusive result however Claassen also reported a lack of significant difference in urinary calcium excretion between ovariectomised and sham rats after a 6 week study period using 24 hour urine samples (Claassen, 1996). It is possible that a 9 week study period was insufficient to result in substantial changes to calcium absorption and excretion. There is also considerable inter-individual variability in calcium excretion which was apparent in both the current study as well as that of Claassen (Claassen, 1996). This may have masked any effect of ovariectomy on calcium excretion.

4.1.2 Effect of EPA Supplementation

To date there are no other reports as to the effects of pure EPA supplementation on either intestinal calcium absorption or renal calcium reabsorption. Increased membrane fluidity as a result of increased incorporation of polyunsaturated fatty acids is believed to upregulate both active and passive calcium transportation across membranes (Kruger & Horrobin, 1997). Increased membrane incorporation of arachidonic acid and increased levels of PGE_2 have been implicated as possible drivers for the rapid increase in intestinal calcium absorption and decrease in renal calcium reabsorption as a result of vitamin D activation (Kruger & Horrobin, 1997). As dietary intake of n-3 fatty acids results in displacement of n-6 EFAs in membrane phospholipids (Watkins *et al,* 1997; Atkinson *et al,* 1997; Watkins *et al,* 2000; Watkins *et al,* 1996), EPA supplementation could be expected to result in displacement of arachidonic acid from membranes. This, in conjunction with decreased PGE_2 production as a result of both decreased substrate availability and n-3 EFA-mediated inhibition of COX-2 activity may lead to decreased intestinal calcium absorption and increased renal calcium reabsorption. However one study has reported increased Ca^{2+} ATPase activity as a result of supplementation with combinations of GLA, EPA and DHA with the greatest effect being evident with a

relatively high ratio of EPA+DHA:GLA (Kruger & Horrobin, 1997). In light of this report, it could be expected that EPA supplementation would increase intestinal calcium absorption and renal calcium reabsorption resulting in increased calcium balance and increased mineralisation of bone. This latter theory is probably a more logical mechanism of action from a physiological perspective as there can be little perceived benefit to the body of inhibiting dietary calcium intake but stimulating renal calcium reabsorption. The effect (if any) of EPA on intestinal calcium absorption is unclear and should be a focus of future research. What little is known about EPA indicates that EPA supplementation may increase renal calcium reabsorption and therefore decrease urinary calcium excretion. This is supported by findings that fish oil and a combination of fish oil + evening primrose oil have been shown to reduce urinary calcium excretion in animals (Buck *et al*, 1991; Kruger *et al*, 1995). Fish oil or tuna oil fed to growing male rats also resulted in lower urinary calcium concentrations than feeding of corn oil or evening primrose oil (Kruger, 2001). However as fish and tuna oil contain a range of n-3 EFAs it is not possible to conclude whether this effect is due to EPA. Tulloch *et al*, 1994 reported a dramatic reduction in urinary calcium excretion upon feeding of the n-6 EFA, GLA. It appears therefore that both n-3 and n-6 EFAs are capable of reducing urinary calcium excretion however some EFAs may be better than others at promoting this effect. The results of the current study suggest that EPA supplementation may have slightly decreased urinary calcium excretion although differences between groups were not significant therefore results are inconclusive (refer Table 5). In the present study there was considerably more inter-individual variability in calcium excretion when results of the three ovariectomised groups were pooled (data not shown) compared to the sham. This may indicate that the normal mechanisms controlling urinary calcium excretion are dysfunctional in instances of estrogen deficiency. As a result, urinary calcium excretion may not be a good indicator of overall calcium balance in the ovariectomised animal.

Results from human trials indicate that high dietary EFA intake may lead to increased renal phosphate reabsorption (Bassey *et al*, 2000) and studies in diabetic rats have shown that supplementation with either DHA or EPA reduces urinary phosphate concentrations (Yamada *et al*, 1995). Results from the present study however suggest that EPA

supplementation had little effect on urinary phosphate concentration. Again this may be a reflection of the considerable inter-individual variability in phosphate excretion encountered and perhaps significant differences may have been evident if a larger number of animals had been tested .

The finding that EPA supplementation did not have a significant effect on either urinary calcium or phosphate excretion may suggest that EPA itself has no effect on membrane transport of calcium and phosphate and the observed increased renal phosphate reabsorption evident in other studies was a result of increased membrane fluidity in general rather than any effect of EPA specifically. In the current study, the percentage of polyunsaturated, monounsaturated and saturated fatty acids in the membranes remained unchanged with EPA supplementation (refer Table 12). It could therefore be expected that membrane fluidity was not significantly altered. As it can not be ruled out that the lack of significant difference in urinary calcium and phosphate excretion between EPA supplemented and unsupplemented groups was not simply a result of a lack of sensitivity in the methodology employed, few conclusions can be drawn from the current study as to the effects of EPA on calcium and phosphate balance.

4.2 Serum C-Telopeptides of Type 1 Collagen

4.2.1 Effect of Ovariectomy

Although serum CTX concentration was higher in the OVX group compared to sham the difference was not significant (refer Table 6). Serum C-telopeptide concentration is indicative of the rate of osteoclastic activity. Osteoclastic activity is increased with estrogen deficiency due to the loss of both the stimulatory effect of estrogen on OPG formation and the inhibitory effect of estrogen on RANK-L formation (Pfeilschifter *et al,* 2002) therefore ovariectomy is expected to raise serum CTX concentrations. Measurement of serum C-telopeptide concentration is a relatively new technique. No other studies involving ovariectomised rats in which CTX concentrations has been used as an outcome measure could be found in the published literature. Other markers of bone resorption include the measurement of type-1 collagen deoxypyridinoline crosslinks (Dpyds) as well

as the measurement of hydroxyproline concentration in urine. Inverse trends between urinary concentrations of both biochemical markers and bone calcium have been reported (Claassen, 1996; Schlemmer *et al*, 1999). Significantly higher urinary hydroxyproline concentrations (Schlemmer *et al*, 1999) and Dpyd concentrations (Kruger *et al*, 1999) have been found in ovariectomised compared to sham rats. However significant differences have not always been apparent when urinary Dpyd concentration has been measured especially in studies of relatively short duration (6 weeks) (Claassen, 1996; Schlemmer *et al*, 1999).

The lack of significant difference in serum CTX concentrations with ovariectomy in the current study may have been a result of the relatively short study duration which perhaps was an insufficient period to allow substantial changes in osteoclastic matrix degradation to occur. The lack of effect may also indicate that the CTX assay used lacks the required sensitivity to detect the magnitude of change evident with ovariectomy in rodents. Parikka *et al* (2001) proposed that there are two phases to the process of osteoclastic degradation of organic matrix. The first phase involves the action of Matrix Metalloproteinases (MMPs) and results in the release of the C-terminal telopeptides of type-1 collagen as measured by the CTX assay. The second phase involves the action of cysteine proteases and leads to the release of different C-terminal fragments of type-1 collagen which contain deoxypyridinoline crosslinks. Estrogen treatment of mature osteoclasts results in significant inhibition of the action of cysteine proteases but appears to have little effect on the action of MMPs (Parikka *et al*, 2001). Ovariectomy would therefore be expected to stimulate cysteine proteases but not MMPs. The release of MMPs from the bone matrix would increase with increased osteoclastic activity and this would be reflected by the CTX assay however the increased activity of cysteine proteases would not be accounted for by this assay. This may mean that the CTX assay underreports the overall level of osteoclastic bone resorption.

4.2.2 Effect of EPA Supplementation

In the present study, EPA supplementation appeared to further increase the breakdown of type-1 collagen and therefore further stimulated osteoclastic activity. This effect was

particularly evident with the high dose rather than low dose supplement. To date there are no reports that either EPA or any of its metabolites influence OPG or RANK-L expression therefore it seems likely that EPA has stimulated collagen degradation by a RANK-L-independent mechanism. This is further supported by the finding that only small increases in RANK-L levels are required for maximal RANK-L stimulated osteoclast activity (Pfeilschifter *et al,* 2002). As estrogen deficiency indirectly results in increased RANK-L concentrations through reduced activation of TGF-β (Pfeilschifter *et al,* 2002) it is unlikely that the addition of another promoter of RANK-L synthesis would result in significant increases in RANK-L stimulated bone resorption. It has also been found that EPA does not inhibit osteoclast formation (Watkins *et al,* 1997) suggesting that EPA does not act to prevent bone mineral or matrix resorption. Few studies have been published which have focused on the effects of pure EPA on collagen synthesis or degradation. From the studies that have been undertaken, there is evidence that EPA specifically promotes collagen synthesis (Watkins *et al,* 2001) and that n-3 EFAs in general decrease the activity of aggrecanase and collagenase (Watkins *et al,* 2003). In the present study, EPA treatment appears to have stimulated collagen degradation as serum CTX concentration was higher in the supplemented groups, although not significantly so, than the OVX controls. The study reporting an inhibitory effect of n-3 EFAs on collagenase and aggrecanase activity was conducted *in vitro* and used a combination of n-3 EFAs (Watkins *et al,* 2003). It is therefore possible that either a combination of n-3 EFAs or an n-3 EFA other than EPA is responsible for this inhibitory effect. As with all *in vitro* studies there is the possibility that the reported effect is not physiologically significant *in vivo.* A subsequent study showed a significant reduction in serum pyridinoline concentration (another marker of bone resorption) in fish oil supplemented ovariectomised rats compared to controls (Li *et al,* 2003). Interestingly, although the fish oil supplemented group had the lowest serum pyridinoline concentration in this study, a group supplemented with DHA had the highest bone mass. DHA supplementation resulted in significantly greater BMD compared to ovariectomised controls but not significant differences in serum pyridinoline concentration. As the bone turnover cycle commences with osteoclastic bone resorption, it would be expected that the effect of a treatment to increase the density of under-mineralised bone would be evident more quickly when the bone turnover rate is high.

4.3 Bone Density

4.3.1 Effect of Ovariectomy

Ovariectomy resulted in substantial decreases in bone mineral content and bone mineral density in both the lumbar spine and femur (refer to Figure 12, Figure 13 & Table 8). This is consistent with the known effects of estrogen deficiency and such a result has been reported in numerous studies involving ovariectomised rats (Jiang *et al,* 1997; Schlemmer *et al,* 1999) and post-menopausal women (Kruger *et al,* 1998; Kruger & Horrobin, 1997; Bassey *et al,* 2000).

4.3.2 Effect of EPA Supplementation

High dose EPA supplementation resulted in further decreases in BMC and BMD (refer Tables 7 and 8) . Low dose EPA supplementation may have had a slight positive effect on BMC and BMD although this effect was not statistically significant. Other studies have reported positive effects of fish oil (van Papendorp *et al,* 1995) and combinations of n-3 and n-6 fatty acids (Kruger *et al,* 1997; Kruger *et al,* 1998; Kruger *et al,* 1999; Schlemmer *et al,* 1999) on bone density in estrogen-deficient animals and humans. It is generally accepted that the ratio of n-6:n-3 EFAs is important and that the two fatty acid families act synergistically to promote bone formation (Horrobin *et al,* 2002). There is some discrepancy as to what the optimal ratio of n-6:n-3 EFAs is and whether perhaps some n-6 and n-3 EFAs are more beneficial than others in promoting bone formation. The diets in the present study contained linoleic acid from corn oil and the n-6:n-3 EFA ratio was approx 2.5:1 in the low dose EPA group and 0.7:1 in the high dose EPA group. Animals exhibited no signs of n-6 EFA deficiency and weight gain in all animals was comparative which again suggests animals were not n-6 EFA deficient. It is likely that a combination of n-3 fatty acids or an n-3 EFA other than EPA is necessary in order to produce a significant, beneficial effect on bone density. It is possible that the high dose EPA supplement inhibited metabolism of linoleic acid by competitive inhibition of Δ6-desaturase (Neumann *et al,* 2003) therefore although the animals were not n-6 EFA deficient per se a lack of some n-6 metabolites relative to n-3 may have been partially responsible for the decreased bone density evident in the high dose EPA group. The high

dose EPA supplement also appeared to inhibit synthesis of EPA metabolites particularly DHA (refer Table 12). Li *et al,* 2003 reported greater increases in BMC and BMD in ovariectomised rats fed DHA compared to those fed fish oil. Inhibition of DHA synthesis by high dose EPA supplementation may therefore be a cause of the reduced BMC and BMD evident in the present study.

In the femoral neck region which is comprised of approximately 75% cortical and 25% trabecular bone and which includes the intertrochanteric area which has a 50:50 ratio of cortical:trabecular bone (Mundy, 1995), high dose EPA supplementation had only a slight, non-significant negative effect on BMC and BMD however ovariectomy resulted in significant decreases in both measures (refer Table 9). In the femur shafts, which are comprised of approximately 95% cortical and 5% trabecular bone, high dose EPA supplementation resulted in significant decreases in BMD however ovariectomy had only a slight negative effect on BMD in this region (refer Table 9). These results indicate that estrogen deficiency appears to have a greater impact on mineral density in bone with a high trabecular component rather than bone with a high cortical component. This is evident by the decrease in BMD of 11.7% in the femoral neck compared to a decrease of just 7.2% in the femoral shaft in the OVX group compared to sham. This finding is consistent with that of a vast array of other studies in both animals as well as post-menopausal women (Baron, 1995; Jiang *et al,* 1997) and further validates the use of the ovariectomised rat as a model for post-menopausal osteoporosis. High dose EPA supplementation resulted in a further 6% decrease in femoral shaft BMD but only a non-significant further 2% decrease in femoral neck BMD.

The percentage decline in BMD due to ovariectomy as well as that due to ovariectomy + high dose EPA supplementation is similar in both femurs and the lumbar spine. Ovariectomy alone produced a 10.0% decrease in femur BMD and a 10.9% decrease in lumbar spine BMD compared to sham. Ovariectomy + high dose EPA supplementation resulted in a 14.6% decrease in femur BMD and a 15.4% decrease in lumbar spine BMD compared to sham. It appears therefore that EPA may exert its effects predominately on cortical rather than trabecular bone. Although the lumbar region of the spine consists of

approximately 66% trabecular bone, the decline in BMD evident in this region in the high dose EPA group may be a result of both a reduction in trabecular BMD due to estrogen deficiency as well as a decrease in cortical BMD due to EPA. It is not possible to measure cortical and trabecular bone density separately using DEXA therefore this theory can not be validated with the available data. pQCT analysis of bones is necessary in order to distinguish between losses of mineral from cortical versus trabecular bone. To date, no studies have been published on the type of bone influenced by EFAs however the finding that EFAs primarily influence cortical bone may explain the apparent discrepancy in results of human trials involving EFA supplementation. Studies in older post-menopausal women (mean ages 79.5-80yrs) have reported positive effects on bone density with EFA supplementation (van Papendorp *et al,* 1995; Kruger & Horrobin, 1997) whereas one study in younger post-menopausal women (50-65yrs) reported no effect (Bassey *et al,* 2000). Loss of bone density due to senescence is largely a result of reduced levels of growth hormone and the effects are evident in cortical rather than trabecular bone (Manolagus & Jilka, 1995; Manolagus, 2000). EFA supplementation may aid in negating the effects of low levels of growth hormone thereby minimizing age-related losses of bone density. n-3 EFA metabolites and low levels of the n-6 metabolite PGE_2 promote IGF and IGFBP synthesis (Watkins & Seifert, 1996). IGF is one of the key growth factors by which growth hormone exerts its effects (Canalis, 1993).

Positive effects of EFA supplementation on bone density have also been reported in various studies involving ovariectomised rats. EPA appears to be beneficial in improving bone strength when calcium intake is inadequate (Sakaguchi *et al,* 1994). Relatively long term supplementation (14 weeks) of ovariectomised rats fed a calcium adequate diet with a 1:3 ratio of evening primrose oil (n-6 EFAs) and fish oil (n-3 EFAs) has also been shown to increase BMD (Kruger *et al,* 1999). EFAs are able to potentiate the effects of exogenously administered estrogen in ovariectomised rats therefore are able to further increase bone mineralisation above that of estrogen or EFA supplementation alone (Schlemmer *et al,* 1999). EFA supplementation is also able to combat the negative effect of impaired insulin levels or activity on bone metabolism therefore appears to be beneficial in preventing the reduction in bone density evident in diabetes (Yamada *et al,* 1995).

Most of the studies reporting a positive effect of EFAs on bone mineralisation have utilized a combination of n-3 and n-6 EFAs. Only two studies, both conducted by Sakaguchi *et al,* have examined the effects of EPA alone and neither of these studies used bone density as an outcome. One of these studies was conducted *in vitro* and demonstrated that EPA stimulates the accumulation of calcium in osteoblasts and that inhibition of PGE_2 synthesis promotes mineralisation (Sakaguchi *et al,* 1994). As the high dose EPA supplement resulted in a significant decrease rather than an increase in bone mineral density in the present study, other factors must have been operating *in vivo* to override this observed *in vitro* effect. The low dose EPA supplement used in the current study resulted in no significant increase in bone mineral density indicating that the calcium sequestering effect of EPA is either not physiologically significant *in vivo* or perhaps not significant under the parameters employed in the current study. Perhaps a dose in between that of the low and high doses used in the present study is required in order for this effect to be apparent *in vivo* or the increase in bone density resulting from this effect may be very gradual and therefore only apparent after a much longer time period than was used in the current study. The second Sakaguchi study on the effects of EPA used bone breaking strength as an outcome measure and is discussed in more detail in section 4.5.2.

4.4 Bone Ash Content

4.4.1 Effect of Ovariectomy

As ovariectomy leads to weight gain and bone mass is usually correlated with body mass it could be expected that ovariectomy would lead to increased bone size and weight. Slight, non-significant increases in dry bone length were evident in all three groups of ovariectomised rats compared to shams however ovariectomy led to a slight decrease in dry bone weight (refer Table 10). Ovariectomy resulted in a significant decrease in the ash content of femurs but the amount of non-mineral bone matter actually increased. DEXA measures of BMC tended to be higher than those obtained from ashing of bones but the calculated % differences in BMC between groups obtained by both methods were similar. This suggests that estrogen deficiency specifically inhibits bone mineralisation and either has no effect or a much smaller effect on formation of the organic matrix. The increase in

body weight resulting from ovariectomy has possibly triggered the increase in matrix formation. As previously discussed, only the cysteine proteases not the metalloproteinases are regulated by estrogen (Parikka *et al,* 2001). Although estrogen deficiency results in increased osteoclastic bone resorption and hence demineralisation of bone, it only increases the rate of the latter phase of collagen degradation not the initial phase (Parikka *et al,* 2001). This may explain the apparent imbalance between organic and non-organic matter which occurred with ovariectomy in the present study. Estrogen deficiency has also been reported to increase the torsional strength of femurs (Jiang *et al,* 1997). Torsional strength is a function of the organic matrix of bone and therefore could be expected to increase in relation to increases in the amount of organic matrix.

4.4.2 Effect of EPA Supplementation

High dose EPA supplementation further decreased bone mineralisation but the majority of its effect appeared to be on the organic matrix where it may have either inhibited formation or promoted degradation. Although results were non-significant and therefore inconclusive, ovariectomy appeared to result in an imbalance in the ratio of non-mineral:mineral bone matter. EPA supplementation may have resulted in a re-dress of this balance as is evident by the ratios of non-mineral:mineral bone matter for the high dose EPA and sham groups being similar. No other studies could be found which have examined the effects of EPA or n-3 EFAs in general on the ratio of mineral:non-mineral bone matter.

4.5 Bone Breaking Strength

4.5.1 Effect of Ovariectomy

Ovariectomy resulted in a significant decrease in the amount of stress bone can absorb before breaking (refer Table 11). Other studies have reported similar findings (Jiang *et al,* 2002).

4.5.2 Effect of EPA Supplementation

Low dose EPA supplementation had a slight but non-significant positive effect on bone strength whereas high dose EPA supplementation had no additional effect on top of that of ovariectomy on most parameters of bone strength. This indicates that although both bone mineral content and density were lower in the high dose EPA supplemented group compared to OVX controls, the mineral loss was not sufficient to result in a physiologically significant effect on the mechanical properties of the bone. Ovariectomy resulted in an increase in bone elasticity which was reversed by both high and low dose EPA supplementation. Bone elasticity is a function of the relative amounts of organic and non-organic matter in bone. Results of bone ashing indicated that unsupplemented, ovariectomised rats had a higher proportion of non-mineral matter in their bones than EPA supplemented rats therefore it would be expected that the bones of ovariectomised rats would be more elastic than those of EPA treated rats.

Sakaguchi *et al* (1994) used a similar dosing regime (0.15g EPA/kg body weight) to that employed in the low dose EPA group (0.1g EPA/kg body weight) but reported a positive effect on bone weight and breaking strength in ovariectomised rats receiving a low calcium diet. There were several differences between this study and the current study which may explain the difference in significance of the reported effects of treatment. Probably of most importance are the different outcome measures used, different controls and different calcium contents of the diets. The Sakaguchi study used a diet with a low calcium content (0.01%) whereas the current study used a calcium-sufficient diet (0.5%). EPA may have a beneficial effect on calcium balance and therefore bone mineralisation when dietary calcium intake is inadequate but it may not have such an effect when calcium intake is adequate. Sham rats were also not included as a control in the Sakaguchi study therefore it is not possible to ascertain the extent to which the EFA supplement helped correct the losses in bone weight and breaking strength resulting from estrogen deficiency. Whether the EFA supplement resulted in a physiologically significant rather than just a statistically significant effect can not be determined.

4.6 Plasma Lipid Content

Due to budget constraints, plasma lipid composition was analysed only at one time point and only in the OVX control and two EPA supplemented groups. As all animals were fed the same diet for the four weeks prior to commencement of the trial and as other studies have shown that the composition of dietary fatty acid is rapidly reflected in the composition of plasma membrane lipids (Watkins *et al,* 1997; Atkinson *et al,* 1997; Watkins *et al,* 2000; Watkins *et al,* 1996), it is assumed that the composition of OVX plasma lipids is indicative of that of the low and high dose EPA groups at the commencement of the supplementation period. EPA supplementation resulted in significant changes in the fatty acid composition of plasma lipids (refer Table 12 and Figure 17). Previous studies have reported that increased dietary intake of n-3 fatty acids results in displacement of n-6 fatty acids from plasma membranes and vice versa (Watkins *et al,* 1997; Atkinson *et al,* 1997; Watkins *et al,* 2000; Watkins *et al,* 1996). However the results from the present study suggest that EPA supplementation does not simply result in a 1:1 replacement of arachidonic acid with EPA in plasma lipids as losses of AA were disproportionately higher than the increases in EPA in plasma lipids for both EPA supplemented groups. DPA and DHA are both metabolites of EPA. However increased EPA content of plasma lipids did not result in proportional increases in either plasma lipid DPA or DHA content (refer Table 12). The low dose EPA group had a 2000% higher concentration of EPA in plasma lipids compared to the OVX group but only a 280% higher DPA content and a 6% higher DHA content. The high dose EPA group had a 9500% greater concentration of EPA in plasma lipids compared to the OVX group but plasma lipid DPA concentration was only 670% higher and DHA concentration was actually 46% lower than OVX controls. This suggests that even the low dose EPA supplement provided more dietary EPA than could be metabolized by the body. Inhibition of DHA synthesis as a result of high dose EPA supplementation (12g EPA/day) has also been reported in a human study (Horrobin *et al,* 2003) suggesting DHA concentration is tightly regulated. EPA conversion to DPA is catalysed by an elongase. Conversion of DPA to DHA requires the activity of elongase, Δ6-desaturase and the mixed function oxidases involved in β-oxidation. EPA supplementation appears to have resulted in inhibition of some or all of these enzymes. The effects of EPA on the activity of Δ 6-desaturase have been studied

(Neumann *et al,* 2003) however little is known about the effects of EPA on function of the other two enzyme systems.

Inhibition of Δ 6-desaturase

Δ 6-desaturase catalyses the desaturation of linoleic (18:2n-6) and α-linolenic (18:3n-3) acids as well as tetracosatetraenoic (24:4n-6) and tetracosapentaenoic (24:5n-3) acids. It has a greater specificity for the 18-carbon compared to the 24 carbon fatty acids and its activity is dependent on the total amount of polyunsaturated fat in the diet. Enzyme activity reaches a maximum when polyunsaturated fat represents <4% dietary energy (Neumann *et al,* 2003). In the present study, all diets provided approximately 4% energy from polyunsaturated fats. The high dose EPA diet provided more energy from polyunsaturated fat (approx 7%) than the other two diets (approx 5% control and 6% low dose EPA). A slight but non-significant increase in the α-linolenic acid content of plasma lipids was evident in the high dose EPA group possibly indicating slightly reduced Δ 6-desaturase activity (refer Table 11). Inhibition of Δ 6-desaturase due to the increased PUFA content of the high dose EPA diet may explain some of the reduction in DHA concentration of plasma lipids seen in this group however it is unlikely to be the main cause of the difference due to the relatively small difference in PUFA content between all three diets. It is possible that the high dose EPA supplement provided an excess of EPA such that the enzymes responsible for its metabolism were simply saturated with substrate, hence the apparent lack of EPA metabolites compared to EPA. This may explain the apparent lack of activity of elongase however as DHA concentration in plasma lipids was lower in the high dose EPA group compared to the other groups, it is theorized that high concentrations of EPA specifically inhibit DHA synthesis via inhibition of β-oxidation. This theory is discussed in more detail in section 4.10.2.

4.7 Vitamin D_3 Metabolites

Due to budget constraints, serum concentrations of only one vitamin D metabolite (25(OH) vitamin D_3) were measured.

4.7.1 Effect of Ovariectomy

Ovariectomy had no significant effect on serum concentrations of 25(OH) vitamin D_3 (refer Table 13). Although some postmenopausal, osteoporotic women have lower serum concentrations of both 25(OH)vitamin D_3 and 1,25(OH)2 vitamin D_3 compared to non-osteoporotic controls (Landin-Wilhelmsin *et al,* 1999), in the majority of cases serum concentrations of vitamin D metabolites are normal (Bikle, 1994). Estrogen deficiency results in decreased binding of Vitamin D to the Vitamin D Receptor (VDR) in intestinal membranes (Leonard *et al,* 2001) and leads to marked resistance to 1,25(OH)2 vitamin D_3 activity as evident by reduced intestinal calcium absorption (Gallagher *et al,* 2001). This latter effect may be due to reduced calbindin-D28k gene expression (Criddle *et al,* 1997). Calbindin is an intra-cellular calcium-binding protein which facilitates calcium absorption (Stipanuk, 2000). Its expression is largely governed by 1,25(OH)2 vitamin D_3 activity (Stipanuk, 2000) however estrogen has been shown to have a direct, stimulatory effect on calbindin gene expression (Criddle *et al,* 1997) and both estrogen and vitamin D appear to be required for maximal gene expression. Estrogen treatment of ovariectomised rats results in increased expression of VDR mRNA as well as increased binding of 1,25(OH)2 vitamin D_3 to the receptor (Liel *et al,* 1999) therefore estrogen may potentiate the actions of vitamin D. Although levels of vitamin D metabolites may be normal, decreased responsiveness to those metabolites may contribute to the pathogenesis of post-menopausal osteoporosis. Vitamin D supplementation even of vitamin D replete post-menopausal women results in increased bone density (Gallagher *et al,* 2001). This provides further support for vitamin D as an agent in the pathogenesis of osteoporosis.

4.7.2 Effect of EPA Supplementation

Only one study involving ovariectomised rats has explored the possible interaction between EFAs and vitamin D. Leonard *et al* (2001) reported that a 1:3 ratio of GLA:EPA+DHA resulted in decreased vitamin D receptor concentrations in intestinal membranes. The effect of EFAs on the relative levels of vitamin D metabolites was not studied.

In the current study, both low and high dose EPA supplementation regimes resulted in significant elevation of serum 25(OH) vitamin D_3 levels. Assuming the serum 25(OH) vitamin D_3 levels of the sham and OVX groups were normal for rats of this age, it appears that EPA supplementation can either increase the synthesis of 25(OH) vitamin D_3 or decrease its conversion to $1,25(OH)_2$ vitamin D_3. Serum concentration of $1,25(OH)_2$ vitamin D_3 was not measured in this study therefore it is not possible to determine the cause of the elevated serum 25(OH) vitamin D_3. As outlined in section 4.10.2 it is theorized that EPA inhibits 1α-hydroxylase activity thereby preventing normal activation of vitamin D_3 and hence resulting in elevated serum 25(OH) vitamin D_3 levels.

4.8 Statistical Correlations

It is recognized that a statistical correlation does not necessarily equate to a physiologically important correlation. The aim of this analysis was to identify the relationship between the various outcomes measured in order to propose possible explanations for the results obtained and identify potential future areas of research.

4.8.1 Relationship between Outcome Measures

Bone ash was positively correlated with bone mineral density as measured by DEXA (refer Table 14). Similar findings have been reported in other studies (Brown, 2001) which further validates the use of DEXA as a tool for measuring bone mineral density.

There was no correlation evident between serum CTX concentration and DEXA bone mineral density, bone ash or organic bone matter content. As discussed in Section 4.2, serum CTX concentration may not be a sensitive marker of osteoclastic activity.

Urinary calcium concentration was positively correlated with urinary phosphate concentration (refer Table 14). Excretion of both ions is differentially regulated by various hormones (Stipanuk, 2000) therefore a correlation between urinary concentration of these ions would not necessarily occur. Only one published study could be found in which urinary calcium and phosphate concentrations were measured and in this study there was

no correlation between urinary concentrations of the two ions (van Papendorp *et al*, 1995). As hormone levels were not measured in the present study, the significance if any of this effect can not be determined.

4.8.2 Organic Bone Matter and EFAs

The percentage of n-3 EFAs in plasma lipids was negatively correlated and n-6 EFAs positively correlated with the amount of organic bone matter in femurs (refer Table 16). Few inferences can be drawn from this however as the high dose EPA supplement resulted in significant bone resorption which would naturally equate to a reduction in organic bone matter. As will be discussed in Section 4.10.1, the reduced bone density evident in the high dose EPA group is believed to be a function of the high proportion of unsaturated fatty acids in this supplement rather than a direct effect of EPA itself. It is likely therefore that the apparent statistical correlation evident in the present study between EFA family and amount of organic bone matter is simply a reflection of the EPA "overdose" indirectly promoting bone resorption. To date there is no evidence in the literature that n-3 EFAs have a catabolic role in bone metabolism (Watkins *et al,* 1997).

4.8.3 Bone Mineral Density and EFAs

No significant correlations between plasma EFA levels and either DEXA bone mineral density or bone ash were apparent in the present study (refer Table 16). Kruger, 2001 reported that DHA may have a role in promoting bone mineralisation. Most of the studies reporting a positive effect of dietary EFAs on bone density have used a combination of GLA, EPA and DHA (Kruger *et al,* 1998; Watkins *et al,* 2000; van Papendorp *et al,* 1995; Kruger & Horrobin, 1997; Schlemmer *et al,* 1999; Kruger *et al,* 1997; Claassen *et al,* 1995). Although not statistically significant, a possible trend was evident in the current study between both DEXA bone mineral density and bone ash and the ratio of GLA:DHA in plasma lipids. It is possible that both GLA and DHA have roles in regulating bone mineralisation.

4.8.4 Vitamin D_3

In the present study serum 25(OH) vitamin D_3 concentration was inversely correlated with femur BMD (refer Table 14). Whilst $1,25(OH)_2$ vitamin D_3 has both a catabolic and an anabolic effect on bone, there is evidence that 25(OH) vitamin D_3 has a purely catabolic effect (Norman & Henry, 1993). However as there was no significant difference in 25(OH) vitamin D_3 concentration between the low and high dose EPA groups in the current study but significant differences in bone density were evident between these groups, there is no evidence from the results of the present study that significant catabolism of bone by 25(OH) vitamin D_3 occurred. If the elevated 25(OH) vitamin D_3 concentration evident in the two EPA supplemented groups is a result of inhibition of $1,25(OH)_2$ vitamin D_3 formation, the inverse correlation between 25(OH) vitamin D_3 concentration and BMD may reflect a lack of $1,25(OH)_2$ vitamin D_3 activity. Again however, as serum concentration of 25(OH) vitamin D_3 was not significantly different between the low and high dose EPA groups it is unlikely that $1,25(OH)_2$ vitamin D_3 concentration differed therefore the decreased bone density evident in the high dose EPA group is unlikely to have resulted from any effect of vitamin D metabolites.

Significant associations between plasma lipid composition and levels of 25(OH) vitamin D_3 were evident. There was no significant correlation between the ratio of n-3:n-6 EFAs and 25(OH) vitamin D3 concentration therefore it appears that vitamin D_3 metabolites selectively change the levels of specific EFAs in plasma lipids (and presumably in lipid membranes). Concentration of 25(OH) vitamin D_3 was negatively correlated with the ratio of AA:DPA, GLA:EPA, and GLA:DPA. Possible negative trends were also evident with the ratios of AA:EPA and GLA:DHA. This suggests that high levels of 25(OH) vitamin D_3 (and presumably low levels of $1,25(OH)_2$ vitamin D_3) are associated with increases in the levels of specific n-3 EFAs in plasma lipids. Correlations between the known functions of $1,25(OH)_2$ Vitamin D_3 and plasma concentrations of the EFAs measured in the present study are outlined in Table 17.

Table 17 Correlation between known functions of 1,25(OH)$_2$ Vitamin D$_3$ and Plasma EFA levels obtained following ovariectomy or sham operation and 9 weeks of feeding a corn oil or EPA supplemented diet.

Functions of 1,25(OH)$_2$ Vitamin D$_3$	**EFAs or ratio of EFAs associated with function**
Increased intestinal calcium and phosphate absorption	Not measured
Increased renal calcium excretion	Unsaturation Index - negative trend with urinary calcium concentration. This may be due to increased levels of EPA and decreased AA. AA is thought to mediate the actions of 1,25(OH)$_2$ vitamin D$_3$ (Baggio, 2000) & is less unsaturated than EPA.
Increased renal phosphate excretion	% DHA - positive trend.
Increased bone mineralisation	GLA:DHA – positive trend with bone density and bone ash. AA:DHA – positive trend with bone density EPA:DHA – negative trend with bone density AA:EPA - negative trend with bone density % EPA – negative trend with bone density Unsaturation Index – negative trend with bone density. This may indicate loss of vitamin D function which is mediated by AA as well as reflecting increased lipid peroxidation.
Increased bone resorption	% GLA – negative trend with serum CTX GLA:EPA – negative trend with serum CTX GLA:DPA – negative trend with serum CTX GLA:DHA – negative trend with serum CTX Unsaturation Index – positively correlated with serum CTX. This is probably just a function of the ratio of GLA to EPA as EPA is more unsaturated than GLA.

It is has been proposed that one of the means by which $1,25(OH)_2$ vitamin D_3 exerts its effects is by altering the EFA composition of membranes (Baggio, 2002) however there is little information to date as to the relationship between EFAs and $1,25(OH)_2$ vitamin D_3 activity. The statistical correlations found in the present study between plasma concentrations of various EFAs and known $1,25(OH)_2$ vitamin D_3 activities may imply that specific EFAs are involved in mediating the actions of 1,25(OH) vitamin D_3. This, coupled with the finding from the present study that EPA supplementation appeared to inhibit $1,25(OH)_2$ vitamin D_3 formation (discussed in Section 4.7.2) suggests that EFAs may not only mediate the action of vitamin D but may also modulate vitamin D activity. This is discussed in more detail and a possible mechanism of action proposed in Section 4.11.

4.8.5 Possible Roles of other EFAs in the Regulation of Bone Metabolism

Other studies have demonstrated a positive effect of fish oil or n-3 EFA supplementation on bone density in estrogen deficient states (Kruger *et al,* 1998; Watkins *et al,* 2000; van Papendorp *et al,* 1995; Kruger & Horrobin, 1997; Schlemmer *et al,* 1999; Kruger *et al,* 1997; Claassen *et al,* 1995). The findings from the present study suggest that EPA is not the main agent responsible for this effect. The following inferences as to the roles of various EFAs are based on statistical correlations between the concentrations of the EFAs in plasma lipids and observed outcomes in this study (refer Table 15).

4.8.5.1 Gamma-Linolenic Acid (GLA)

$1,25(OH)_2$ vitamin D_3 is known to be required for the effective mineralisation of the bone matrix. From the statistical correlations evident in the present study it is possible that GLA has a role in vitamin D mediated bone mineralisation. Percentage GLA in plasma lipids was negatively associated with serum CTX levels suggesting that GLA may have an anti-resorptive effect. These findings are supported by those of several other studies which have reported increased bone mineralisation and decreased resorption when supplements with higher ratios of GLA:EPA(Watkins *et al,* 2000; Claassen *et al,* 1995) and GLA:EPA+DHA (van Papendorp *et al,* 1995; Kruger & Horrobin, 1997; Kruger *et al,* 1997) are administered to animals and humans. Other studies have indicated that EPA does

not have an anti-resorptive effect (Watkins *et al,* 1997) which further validates the theory that GLA may be responsible for this effect.

4.8.5.2 Docosahexaenoic Acid (DHA)

High intakes of EPA appear to inhibit formation of DHA and concentration of DHA in membrane lipids appears to be under tight regulation and is maintained at a relatively low level (McLennan, 2003; Horrobin *et al,* 2003). This degree of regulation suggests that DHA has an important biological function. Plasma lipid DHA content was positively correlated with urinary phosphate excretion (refer Fig 32). Urinary phosphate excretion is under the control of various hormones including PTH, $1,25(OH)_2$ vitamin D_3 both of which promote urinary phosphate excretion and calcitonin and insulin which stimulate renal phosphate reabsorption (Kronenberg, 1993; Stipanuk, 2000). DHA may mediate the effects of some or all of these hormones.

4.9 Context

It is worth noting that the high dose EPA supplement used in this study delivered a pharmaceutical dose of EPA and this is considerably greater than would be feasibly consumed by a human from normal dietary sources. Different fish oils contain varying amounts of EFAs and different fish contain varying amounts of lipid. For instance hoki, one of the cheapest and most readily available fresh fish in New Zealand, contains on average 0.03% EPA (Seafood Division, Crop & Food Research, NZ). A 60kg person would therefore need to consume 20kg of hoki per day in order to receive 1g EPA/kg body weight. Silver warehou is one of the richest sources of EPA with EPA constituting on average 0.63% by weight (Seafood Division, Crop & Food Research, NZ). Even with this rich source of EPA, a 60kg person would need to consume 952g of warehou every day in order to match the intake of the high dose EPA group in the current study. It should also be noted that fish lipids are comprised of EFAs other than just EPA and that humans typically consume a mixed diet containing a higher ratio of n-6:n-3 EFAs. It is therefore logically sound that the high dose EPA supplement could have had such a deleterious effect on bone metabolism as reported in the current study as there is no physiological need for the body

to have developed a mechanism by which to prevent such a high EPA intake from disrupting the normal calcium control system.

Acute, sub-acute and chronic toxicity studies have been conducted on EPA ethyl ester in rats (Shibutani *et al,* 1989; Shibutani *et al,* 1989*; Shibutani *et al,* 1989#). From these studies, the no-effect dose of EPA ethyl ester was estimated to be 0.1g/kg (Shibutani *et al,* 1989). Increased alkaline phosphatase activity possibly indicative of liver injury was evident in animals fed 1.0g/kg body weight for 12 months (Shibutani *et al,* 1989) but no effect was evident after 90 days of feeding (Shibutani *et al,* 1989#). A dose of 3.0g/kg resulted in a decrease in the number of thrombocytes as well as damage to the tail and skin around the anus due to leakage of an oily substance from the anus. At a dose of 6.0g/kg animals showed decreases in body weight, food consumption and α1-globulin and increases in lymphocytes and γ-globulin (Shibutani *et al,* 1989#). Observations from the present study in which the high dose EPA supplement resulted in reduced bone density indicate that 1.0g EPA/kg body weight is a toxic dose in instances of estrogen deficiency.

SECTION 2

Proposed Mechanisms

Two mechanisms are proposed as possible explanations for the results obtained in the present study. The first mechanism relates specifically to the observed effects of the high dose EPA supplement. The second mechanism outlined is a possible means by which EFAs as localised regulators of bone metabolism interact with systemic regulators.

4.10 High Dose EPA Supplement and Calcium Handling

From the results obtained in the current study it is not possible to deduce the mechanism by which the high dose EPA supplement caused the observed reduction in bone density. The following explanation is put forward and is based on the assumptions that:

1. Serum calcium balance was compromised in the high dose EPA group.
2. EPA inhibits the function of mitochondrial mixed function oxidases.
3. Loss of bone density in the high dose EPA group was a result of the PTH-mediated stimulation of bone resorption.

The reasoning behind these assumptions is outlined in more detail in the following section.

4.10.1 High Dose EPA Supplementation Resulted in Calcium Deficiency.

The loss of bone density in the high-dose EPA supplement group as measured by DEXA was relatively large and occurred very rapidly - a 5% decrease in bone density was observed after just 9 weeks of dietary supplementation. As the bone turnover cycle of a rat is approximately 24 days in duration, this loss occurred after only two complete resorption/formation cycles at distinct and probably isolated sites within the skeleton. EFAs and their metabolites are localized regulators of bone metabolism (Kruger & Horrobin, 1997). It is unlikely therefore that the EFAs themselves have directly triggered the increase in osteoclastic activity. Instead it is more likely that the high dose EPA

supplement has caused a major disruption in calcium balance in the body which has lead to promotion of osteoclastogenesis.

There are five possible ways in which a low serum calcium concentration could arise:

1. Inadequate dietary calcium intake.
2. Increased urinary calcium excretion.
3. Reduced intestinal calcium absorption.
4. Excessive bone mineralisation.
5. Ectopic calcification ie calcium is deposited in soft tissue rather than bone.

In the present study dietary calcium intake was adequate, there was no evidence of increased urinary calcium excretion in the EPA supplemented groups and level of bone mineralisation decreased rather than increased with the high dose EPA supplement, it is proposed that high dose EPA resulted in reduced intestinal calcium absorption and may have led to the development of ectopic calcification.

4.10.1.1 Effect of High Dose EPA on Intestinal Calcium Absorption

It is proposed that the high dose EPA supplement led to inhibition of intestinal calcium absorption due to the formation of lipid peroxides in intestinal membranes. As the EPA supplement was stored under nitrogen and diets were made up fresh every day, it is not believed that the EPA was oxidised prior to feeding. The degree of membrane lipid peroxidation was not measured nor was the level of faecal calcium excretion however this theory is based on the following observations:

1. In other studies, the unsaturation index has been shown to be positively associated with activity of membrane-associated calcium transporters (Schlemmer *et al,* 1999; Kruger, 2001). Calcium absorption and the activity of calcium transporters was not measured in the present study however a negative correlation was evident between the unsaturation index and both urinary calcium excretion and bone mineral density and a positive correlation was evident with serum CTX concentration (refer Table 15). These three results suggest that metabolic processes were acting to correct a

serum calcium deficit and that this deficit was possibly related to the degree of membrane unsaturation. As dietary calcium intake was the same across all four study groups, it is proposed that a reduction in intestinal absorption must have occurred in the high dose EPA group. As discussed in section 4.7.2, EPA may inhibit synthesis of $1,25(OH)_2$ vitamin D_3 and therefore could indirectly inhibit vitamin D-dependent calcium transport. However as there was no significant difference between the low dose and high dose EPA groups in terms of serum 25(OH) vitamin D_3 concentrations, inhibition of vitamin D-dependent calcium transport can not be the mechanism responsible.

2. The unsaturation index in the high dose EPA group was considerably higher than in the other groups (refer Table 12).
3. As the polyunsaturated fat content of lipids increases, their susceptibility to peroxidation also increases as the protective capacity of available antioxidants and antioxidant enzymes is exceeded (Stipanuk, 2000). The degree of fatty acid unsaturation is the key determinant of susceptibility to oxidation. Peroxidation of polyunsaturated fatty acids in lipid membranes may result in decreased membrane fluidity, membrane depolarization (Lu *et al,* 2001) and as a result could feasibly lead to decreased calcium transport. To date there are no reports as to the effects of membrane lipid peroxidation on intestinal absorption of nutrients however one study has reported that hydroxyl radical inhibits Ca^{2+}-ATPase function in heart and skeletal muscle by directly inhibiting ATP binding (Xu *et al,* 1997). Lipid peroxidation results in the generation of free radicals (Stipanuk, 2000). It also leads to accumulation of free radicals generated by other metabolic processes as a result of saturation of endogenous antioxidant systems. It is therefore thought feasible that membrane peroxidation could lead to accumulation of reactive oxygen species (ROS) that in turn lead to reduced intestinal calcium absorption. Faecal calcium content was not measured in this study but it is possible that faecal calcium concentration would have been higher in the high dose EPA group than in all other groups.

4.10.1.2 Ectopic Calcification

Ectopic calcification is another possible factor which may have contributed to reduced serum calcium concentrations and bone mineral content in the high dose EPA group. Oxidised fatty acids have been found to inhibit differentiation of bone cells and promote calcification of vascular cells in cell culture (Parhami *et al,* 1997). Lipid oxidation products have been postulated as being a possible cause behind the often concurrent occurrence of atherosclerosis and osteoporosis (Parhami *et al,* 1997). The bacterial cell wall contains lipids which are oxidised by agents released by macrophages such as superoxide radical. The presence of oxidised lipids may trigger the immune system which responds to inflammation in soft tissue by calcifying the surrounding tissue and therefore sealing the affected area from the rest of the body (Demer, 2002). If the assumption that the high dose EPA supplement led to increased lipid peroxidation is correct then some ectopic calcification may have occurred .

EFAs and/or their metabolites are known to regulate gene expression of various enzymes via the action of the transcription factors, PPARs (Stipanuk, 2000; Price *et al,* 2000) (refer section 2.2). There are three types of PPARs known as alpha, beta and gamma with the gamma group being further divided into two sub-types designated γ-1 and γ-2. Different types of PPARs predominate in different tissues. For instance PPAR-α is found in high concentrations in tissues such as the kidney proximal tubule where a high level of mitochondrial and peroxisomal β-oxidation occur. PPAR-β is the most abundant and ubiquitously expressed of the three transcription factor types. It is found in the nervous system, skeletal and cardiac muscle, placenta and large intestine. PPAR-γ expression is mainly restricted to white and brown adipose tissue and the intestinal mucosa (Desvergne *et al*, 1999). PPARs bind to Retinol-X-receptors (RXR) to form an active transcription factor. The PPAR-RXR complex can interact with either specific Peroxisome Prolifereator Response Elements (PPREs) or Estrogen Response Elements (EREs) in target genes (Desvergne *et al,* 1999). PPAR-γ inhibits osteoblastogenesis and promotes differentiation of existing osteoblasts into adipocytes (Diascro *et al,* 1998). Linoleic acid peroxidation products have been found to stimulate expression of PPAR-γ (Corwin, 2003). n-3 EFA peroxidation products may have a similar stimulatory effect on PPAR-γ expression which

may help explain the further increase in bone resorption relative to formation evident in the high dose EPA group compared to the ovariectomised controls.

4.10.2 EPA inhibits Mitochondrial Mixed Function Oxidases.

Although not one of the classical functions usually associated with EFA activity, there is some evidence that EPA may inhibit the activity of mixed function oxidases.

1. From the results in the present study, both low and high dose EPA supplementation appear to have inhibited the formation of $1,25(OH)_2$ vitamin D_3 from 25(OH) vitamin D_3 (refer Table 13) and the formation of DHA from EPA (refer Table 12). Coincidentally, mixed function oxidases are a common element in both metabolic pathways.
2. n-3 EFAs increase insulin sensitivity possibly indirectly as a result of increased intracellular Ca^{2+} (McLennan, 2003). This finding is supported by studies in both rats and elite male cyclists which have reported decreased oxygen consumption and heart rate for a given work output after fish oil supplementation (McLennan, 2003). The most likely explanation is an n-3 fatty acid-induced change in the respiratory quotient due to stimulation of insulin activity resulting in increased utilization of glucose as a fuel source. Insulin is known to inhibit β-oxidation (Stipanuk, 2000). n-3 EFAs may either directly inhibit β-oxidation or may indirectly cause inhibition due to their action in modifying the cell response to insulin. The β-oxidation pathway also involves mixed function oxidases.
3. EFAs regulate transcription of certain enzymes via the action of PPARs. One of the PPAR-regulated enzymes is cytochrome P450, a mixed function oxidase involved in β-oxidation of fatty acids (Stipanuk, 2000). EPA promotes synthesis of PPAR-γ1 which is a RXR-dependent nuclear transcription factor (Desvergne & Wahli, 1999). An inverse relationship between the expression of PPAR-γ and cytochrome P450 mRNA has been observed in the rat ovary (Komar & Curry, 2003). Estrogen deficiency results in the loss of inhibition of NF-κB expression and may lead to increased production of Reactive Oxygen Species (ROS) as estrogen also has an antioxidant role (Pfeilschifter *et al,* 2002). Both of these factors result in down-

regulation of PPAR-α expression. (Cabrero *et al,* 2002) PPAR-α appears to be important in stimulating P450 activity in response to xenobiotics (Savas *et al,* 1999).

EPA may also inhibit enzyme activity by the following means:

1. Disruption of the electrochemical gradient.

   The activity of mitochondrial mixed function oxidases is disrupted by alterations in the electrochemical gradient across the mitochondrial membrane. This may occur simply as a result of the physical incorporation of EPA into membranes and the resultant displacement of other membrane fatty acids. This possibly leads to a change in the charge distribution across the membrane due to the difference in polarity of EPA relative to the membrane fatty acids it displaces. n-3 fatty acids have been shown to cause the release of Ca^{2+} from mitochondria resulting in increased cytosolic Ca^{2+} concentration (Stipanuk, 2000). Ca^{2+} membrane transport is coupled to that of Na^{+} which is an important component in the maintenance of electrochemical gradients within cells (Stipanuk, 2000). Release of Ca^{2+} and therefore Na^{+} from mitochondrial membranes may result in sufficient disruption of the mitochondrial electrochemical gradient so as to inhibit the mixed function oxidases involved in β-oxidation.

2. Inhibition of formation of phosphorylated cofactors

   Intracellular Ca^{2+} also acts as a second messenger and has been shown to regulate the synthesis and activity of various enzymes particularly protein kinases which are involved in controlling phosphorylation reactions (Stipanuk, 2000). Changes in the level of intracellular Ca^{2+} may disrupt formation of the phosphorylated cofactors involved in β-oxidation such as NADPH. EFAs also regulate malic enzyme and glucose-6-phosphate dehydrogenase gene expression, both of which are required for the synthesis of NADPH (Stipanuk, 2000).

3. Mitochondrial Uncoupling.

   Recently it has been found that n-3 EFAs induce the expression of two mitochondrial uncoupling proteins in skeletal muscle thereby inhibiting the mitochondrial β-oxidation pathway and promoting peroxisomal fatty acid oxidation

(Price *et al,* 2000). Mitochondrial uncoupling in effect leads to specific inhibition of mitochondrial enzymes. Both $1,25(OH)_2$ vitamin D_3 and DHA synthesis is dependent on mitochondrial mixed function oxidases.

4.10.3 High Dose EPA Supplementation led to PTH-Mediated Bone Resorption.

Inhibition of 1α-hydoxylase could have led to a lack of inhibition on PTH secretion by $1,25(OH)_2$ vitamin D_3 (Kronenberg, 1993). This, coupled with the postulated low serum calcium balance due to inhibited intestinal calcium absorption could have resulted in abnormally high PTH concentrations. Constant, high PTH levels stimulate osteoclast formation and activity leading to bone resorption (Kronenberg, 1993). Thus PTH activity may be responsible for the decrease in bone mineral and matrix content seen in the high dose EPA supplemented group. High PTH levels are associated with high levels of cAMP (Fu *et al,* 2002). High cAMP concentrations stimulate the transcription of collagenase, a key enzyme catalyzing collagen degradation (Partridge & Winchester, 1996). The bone ash and bone elasticity results obtained in this study are consistent with increased collagen degradation in the EPA supplemented groups compared to the ovariectomised controls. Intermittent PTH secretion has an anabolic effect on both trabecular and cortical bone however continuous PTH secretion selectively catabolises cortical bone (Finkelstein, 1996). In the present study high dose EPA supplementation appeared to result in loss of cortical rather than trabecular bone which adds further support to the theory that the loss of bone mineral as a result of high dose EPA supplementation was due to the action of PTH.

4.11 The Essential Fatty Acid/Hormone Interaction Theory

From the available data it appears that both n-6 and n-3 EFAs act to increase calcium balance in the body and both have the potential to increase bone density. Little is known about the mechanism of action of either fatty acid family in regulating bone metabolism however they appear to act by slightly different mechanisms. Both n-6 and n-3 EFAs may

mediate the effects of vitamin D_3. Two observations from studies in patients with idiopathic calcium nephrolithiasis provide support for this theory.

1. A close correlation between plasma PGE_2 levels and serum $1,25(OH)_2$ vitamin D_3 concentration exists (Baggio *et al,* 2000).
2. Fish oil supplementation reduces plasma PGE_2 and serum $1,25(OH)_2$ vitamin D_3 concentration but not 25(OH) vitamin D_3 concentration (Baggio *et al,* 2000).

$1,25(OH)_2$ vitamin D_3 selectively incorporates arachidonic acid into membranes (Baggio *et al,* 2000). This is thought to be at least partially responsible for the rapid increase in intestinal calcium absorption characteristic of vitamin D_3 activity (Baggio, 2002). PGE_2 stimulates 1α-hydroxylase activity thereby promoting further formation of $1,25(OH)_2$ vitamin D_3 from 25(OH) vitamin D_3 (Baggio *et al,* 2000; Baggio, 2002). In this way a positive feedback loop is created. n-6 EFAs and their metabolites (particularly PGE_2) increase intestinal calcium absorption and increase bone resorption both of which rapidly increase serum calcium balance. n-6 EFA metabolites also increase urinary calcium excretion and the process of bone formation (Kruger & Horrobin, 1997). As serum calcium is tightly controlled within a narrow concentration range (Mundy, 1995), these latter functions possibly serve as an "overflow" mechanism ensuring serum calcium does not rise above the set physiological threshold before feedback inhibition mechanisms are activated.

n-3 EFAs inhibit prostaglandin synthesis and also appear to inhibit $1,25(OH)_2$ vitamin D_3 synthesis (Baggio *et al,* 2000; Baggio, 2002). They promote renal phosphate reabsorption (Yamada *et al,* 1995) which further inhibits vitamin D_3 activation (Norman & Henry, 1993). n-3 EFAs therefore inhibit the positive feedback loop involving PGE_2 and vitamin D_3 by decreasing the availability of n-6 EFAs. n-3 EFAs also inhibit the expression of vitamin D receptors (VDR) thereby reducing the sensitivity of cells to vitamin D (Leonard *et al,* 2001). EPA promotes the synthesis of PPAR-γ1, an RXR-dependent transcription factor. The VDR is also dependent on RXR for activity. Direct competition between

PPAR-γ and VDR for RXR may lead to further decreases in VDR activity (Desvergne & Wahli, 1999).

n-3 EFAs and/or their metabolites increase calcium balance by promoting intestinal calcium absorption when dietary calcium intake is low as well as by promoting renal calcium and phosphate reabsorption. This also serves to increase serum calcium balance. n-3 EFAs negate the stimulatory effect of n-6 EFAs on bone turnover but do not appear to directly inhibit bone resorption (Watkins *et al,* 1997). Once the upper threshold for serum calcium balance is reached, the excess calcium and phosphate ions will be deposited in bone resulting in an increase in bone density. It is possible that n-6 EFAs are the "first-line defense" mechanism of $1,25(OH)_2$ vitamin D_3 acting to rapidly increase serum calcium whereas n-3 EFAs function as modulators of n-6 EFA and therefore vitamin D_3 action. n-6 EFAs act to return serum Ca^{2+} levels to normal in response to short term fluctuations in concentration. Whilst $1,25(OH)_2$ vitamin D_3 incorporates arachidonic acid into membranes (Baggio *et al,* 2000; Baggio *et al,* 2000*; Baggio, 2002), EPA incorporation into membranes may not be regulated by vitamin D and may solely be a function of the availability of EPA suggesting enhanced specificity of ligases for EPA rather than AA. Although there are no reports as to the mechanism controlling EPA incorporation into membranes, the finding in the present study that removal of arachidonic acid from membranes was disproportionate to the increase in EPA content of membranes suggests that the two events are under somewhat different control mechanisms.

$1,25(OH)_2$ vitamin D_3 in conjunction with serum calcium, may enhance pancreatic secretion of insulin (Christakos, 1996) which is essential for bone mineralisation. n-3 EFAs increase the sensitivity of cells to insulin and may be important in mediating insulin action. It is therefore evident why the balance between n-3 and n-6 EFAs is important for optimizing bone mineralisation. Excessive levels of n-6 EFAs will lead to high levels of PGE_2 resulting in bone resorption. Excessive levels of n-3 EFAs will inhibit vitamin D action and will also lead to bone resorption. In order to maximize bone formation, the balance between the two EFA families needs to be such that both vitamin D and insulin activity are optimized.

n-6 EFAs and their metabolites form a crucial part of the body's inflammatory response to pathogen attack. One step in this defense system is to "wall off" the pathogen or inflamed tissue from the rest of the body by calcifying the surrounding tissue (Demer, 2002). It is theorized therefore that n-6 EFAs increase serum calcium balance. However once the upper threshold of serum calcium concentration is reached excess calcium is not only deposited in bone but can also occur in ectopic soft tissue. Both fish oil & EPA have been shown to reduce calcium deposition in soft tissue (Das, 2000). It is therefore possible that n-3 EFAs are responsible, either directly or indirectly (perhaps as a result of the requirement for insulin), for diverting excess serum calcium to bone rather than soft tissue.

CHAPTER 5
CONCLUSIONS & FUTURE RESEARCH

5.1 Conclusions

High dose EPA supplementation (1.0g/kg body weight) significantly reduced bone density in ovariectomised rats. It is proposed that the decrease in bone density was an indirect effect of consumption of a diet with a high unsaturation index rather than a result of any specific action of EPA in directly stimulating bone resorption or inhibiting bone formation.

Low dose EPA supplementation (0.1g EPA/kg body weight) had no significant effect on bone density, bone strength, urinary calcium or phosphate excretion or serum concentration of type 1 collagen c-telopeptide compared to the effects of ovariectomy alone. As it cannot be discounted that the lack of significant effect due to low dose EPA supplementation was not simply a result of the relatively short supplementation period in the current study, it cannot be concluded that EPA does not have an effect on bone metabolism.

EPA appears to influence the metabolism of vitamin D_3. It is proposed that EPA inhibits hydroxylation of 25(OH) vitamin D_3 to $1,25(OH)_2$ vitamin D_3 and therefore may regulate vitamin D_3 activation. Various EFAs may also serve as mediators of $1,25(OH)_2$ vitamin D_3 action.

Under the study conditions employed, EPA supplementation did not have a beneficial effect on bone mass following ovariectomy in rodents. The hypothesis that EPA supplementation can prevent the loss of bone resulting from ovariectomy could not be validated by the present study.

5.2 Implications for Future Research

The following areas have been identified as requiring further research:

- The effects of medium dose EPA supplementation (0.5g/kg) on bone turnover.
- The effects of longer term EPA supplementation (between 10 and 14 weeks) on bone turnover.
- The effects of other single EFA supplements particularly GLA and DHA on bone turnover.
- Further studies as to the effect of EFA ratio (particularly the relative ratios of GLA, EPA and DHA) on bone turnover.
- The effect of EPA and other EFAs on activity of mixed function oxidases particularly 1α-hydroxylase.
- The effect of high unsaturated fat intake on membrane lipid peroxidation and intestinal calcium absorption.

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