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

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Abstract

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

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

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Abbreviations

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

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

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