In vitro effects of arachidonic acid and docosahexaenoic acid on osteoclastogenesis and bone resorption in human CD14+ monocytes

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by

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Submitted in partial fulfilment of the requirements of the degree:
Master of Science (Physiology)
in the Faculty of Health Sciences, University of Pretoria

November 2014

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Dedication

I dedicate my dissertation work to my loving parents, Raphael and Nora Kasonga. Their support, encouragement, and constant love have sustained me throughout my life.

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Summary

Bone remodelling is a continuous physiological process in the body mediated by bone resorbing osteoclasts and bone forming osteoblasts. Osteoclasts are multinucleated cells formed by the fusion of haematopoietic cells of monocytic lineage in the presence of receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), which are produced by osteoblasts. Osteoclast over-activity results in increased resorption of bone in some pathological conditions such as osteoporosis. Clinical and animal studies have shown that dietary fatty acids play a role in bone regulation. However there are no reported studies on the effects of long chain polyunsaturated fatty acids (LCPUFAs) on a human osteoclast cell line. The aim of this study was to determine the effects of arachidonic acid (AA), an ω-6 LCPUFA, and docosahexaenoic acid (DHA), an ω-3 LCPUFA, on osteoclastogenesis and bone resorption in human CD14+ monocytes, *in vitro*.

CD14+ monocytes, isolated from human peripheral blood, were seeded on either glass cover-slips or dentine discs in cell-culture plates in the presence of differentiation factors (RANKL and M-CSF). To test the effects of the LCPUFAs on differentiating osteoclasts, the cells were exposed to the LCPUFAs from day 3. For experiments on mature osteoclasts, the cells were exposed to the LCPUFAs from the onset of resorption (day 11-14). All experiments were terminated 7 days after the onset of resorption.

Tartrate-resistant acid phosphatase (TRAP) is an enzyme highly expressed and secreted by mature osteoclasts. The activity of TRAP in culture media and the number of multinucleated TRAP-stained cells were determined. The degree of resorption by mature osteoclasts on dentine was also determined. Cell morphology and actin ring (required for the structural integrity of osteoclasts) formation were also analysed. The expression of prominent osteoclast receptors, vitronectin receptor (VNR) and calcitonin receptor (CTR) were analysed by immunofluorescence. The presence of

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resorptive enzymes (TRAP, MMP-9, cathepsin K) was determined by western blot and the regulation of genes involved in osteoclast formation and activity were assessed by PCR.

Both LCPUFAs decreased osteoclast formation in differentiating osteoclasts resulting in fewer osteoclasts compared to the control. In differentiating osteoclasts, VNR and CTR expression was affected by AA while DHA only affected VNR expression. Both LCPUFAs decreased the expression of all proteins and genes tested in differentiating osteoclasts. AA and DHA were shown to decrease resorption without affecting osteoclast numbers in mature osteoclasts. The integrity of the actin rings formed was unaffected. This may imply that the LCPUFAs have no harmful effect on mature osteoclasts. Both LCPUFAs were shown to affect VNR and CTR expression in mature osteoclasts. AA decreased the expression of all genes and proteins tested in mature osteoclasts, while DHA had no effect on two proteins (MMP-9, TRAP) and two genes (TRAP, CA2).

The results suggest that LCPUFAs can affect osteoclastogenesis and resorption through modulation of osteoclast specific genes. This novel study also demonstrates that the LCPUFAs tested can modulate osteoclast formation and function and may potentially strengthen bones and prevent or delay the onset of osteoporosis.

Keywords: osteoclasts; long chain polyunsaturated fatty acids; bone resorption; arachidonic acid; docosahexaenoic acid; human CD14+ monocytes

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Research Outputs

1. A manuscript was written and will be submitted to *PLOS ONE* for publication.
2. Conference proceedings:
    - Kasonga A, Kruger M, Coetzee M. Arachidonic acid and docosahexaenoic acid inhibits osteoclastogenesis and bone resorption in human CD14+ monocytes, *in vitro*. Presented at the European Calcified Tissue Society Conference ECTS 2014, Prague, Czech Republic. Bone Abstracts Vol 3, PP159 (doi: 10.1530/boneabs.3.PP159)
3. Conference outputs:
    - Kasonga A, Kruger M, Coetzee M. *In vitro* effects of arachidonic acid and docosahexaenoic acid on osteoclastogenesis and bone resorption in human CD14+ monocytes.
        - Oral presentation: Faculty day of the Faculty of Health Sciences, University of Pretoria. August 2014.
        - Poster presentation: Jaarkongres van die Suid Afrikaanse Akademie vir Wetenskap en Kuns: Afdeling Biologiese Wetenskappe. 16 October 2013. *Winner for best poster presentation in MSc category*.
        - Poster presentation: Physiology Society of Southern Africa 41st Conference. 15-18 September 2013.

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Acknowledgements

- I would like to express my deepest gratitude to my supervisor, Dr Magdalena Coetzee without whose encouragement, patience and guidance I would have never completed this project. Special thanks to my co-supervisor, Prof Marlena Kruger, for her advice and support.

- Thanks go out to my colleagues at the Department of Physiology, in particular to Dr Vishwa Deepak whose expertise and assistance helped accelerate my project and to my research associates who have always been accommodating and a pleasure to work with.

- I would also like to thank the Head of Department of Physiology, Prof Dirk van Papendorp, for creating a work environment that supports and motivates academic advancement.

- I would like to thank the medical doctors in the department, Dr Tomek Piorkowski, Dr Prashilla Soma, Dr Craig Grobbelaar and Dr Joji Theron, who were always willing to draw blood for me. Many thanks as well to all the donors who volunteered to give blood for this project.

- A special word of thanks to the Gartland Bone Group, in particular to Karan Shah and Ankita Agrawal, who taught me the techniques needed to culture human osteoclasts, prepare and stain dentine discs, as well as assisted in preliminary experiments conducted at the Mellanby Bone Centre, University of Sheffield, UK. After almost a year of failed attempts to culture human osteoclasts, their assistance was essential in establishing protocols and getting my project started.

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- Thanks go out to the Medical Research Council (MRC) and the University of Pretoria (RESCOM) for funding this study. A special thanks to the UP Postgraduate Studies Abroad Programme for funding the research visit to the Mellanby Bone Centre.

- Thanks to Dr Gerhard Steenkamp (Veterinary Hospital, Onderstepoort), who kindly donated the hippopotamus tusk, and to Prof Willie van Heerden and Mrs Rene Sutherland (Department of Oral Pathology and Oral Biology) who agreed to section the tusk for me so that I could prepare dentine discs. Thanks as well to the Laboratory for Microscopy and Microanalysis of the University of Pretoria who were consulted on microscopic photography of the dentine discs.

- Thanks as well to Marizanne Booyens and Creative Studios, Department of Education Innovation for assisting in preparing several of the illustrations and figures used in this dissertation.

- I would also like to thank my friends, my two elder sisters, my younger brother, but mostly my parents for always supporting and encouraging me even though they have no idea what exactly it is that I do.

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List of Abbreviations and Acronyms

4EBP1	–	Eukaryotic translation initiation factor 4E-binding protein
α-MEM	–	Alpha-minimum essential medium
AA	–	Arachidonic acid [20:4 (5, 8, 11, 14)]
Akt	–	Protein Kinase B
ALA	–	Alpha-linolenic acid [18:3 (9, 12, 15)]
ALP	–	Alkaline phosphatase
ANOVA	–	Analysis of variance
AU	–	Arbitrary unit
BMD	–	Bone mineral density
BMM	–	Bone marrow macrophages
BMP	–	Bone morphogenetic protein
BSA	–	Bovine serum albumin
c-Fms	–	Macrophage colony-stimulating factor receptor
c-Src	–	Tyrosine-protein kinase CSK
CA2	–	Carbonic anhydrase 2
cAMP	–	Adenosine 3’,5’-cyclic mono-phosphate
$C_4H_4Na_2O_6$	–	Sodium tartrate
Cbfa1	–	Core binding factor alpha
CO	–	5% corn oil
$CH_3COONa_2{\cdot}3H_2O$	–	Sodium acetate trihydrate
COX	–	Cyclooxygenase
$cPLA_2$	–	Cytosolic phospholipase A_2
CT	–	Calcitonin
CTR	–	Calcitonin receptor
CTSK	–	Cathepsin K
DC-STAMP	–	Dendritic cell-specific transmembrane protein
DHA	–	Docosahexaenoic acid [22:6 (4, 7, 10, 13, 16, 19)]

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DOC	–	Differentiating osteoclasts
DGLA	–	Dihomo-gamma-linolenic acid [20:3 (8, 11, 14)]
DNA	–	Deoxyribonucleic acid
cDNA	–	Complementary DNA
dNTP	–	Deoxynucleotide triphosphate
EDHA	–	Ethyl docosahexaenoate
EDTA	–	Ethylene-diamine-tetra-acetic acid
eNOS	–	Endothelial NOS
EPA	–	Eicosapentaenoic acid [20:5 (5, 8, 11, 14, 17)]
EphB4	–	Ephrin type-B receptor 4
ERK1	–	Extracellular signal-regulated kinase 1
FA	–	Fatty acid
FBS	–	Foetal bovine serum
FO	–	5% fish oil and 0.5% corn oil
GAPDH	–	Glyceraldehyde 3-phosphate dehydrogenase
GLA	–	Gamma-linolenic acid [18:3 (6, 9, 12)]
H^+	–	Hydrogen ions
$HCON(CH_3)_2$	–	Dimethylformamide
HSC	–	Haematopoietic stem cell
IGF	–	Insulin-like growth factor
IGFBP	–	Insulin growth factor binding protein
IKK	–	Inhibitory kappa kinase
IL-	–	Interleukin-
IL-1R	–	IL-1 receptor
iNOS	–	Inducible NOS
JNK	–	c-Jun N-terminal kinase
LA	–	Linoleic acid [18:2 (9, 12)]
LCPUFA	–	Long chain polyunsaturated fatty acid
LDL	–	Low density lipoprotein
LOX	–	Lipoxygenase
MAPK	–	Mitogen-activated protein kinases
MOC	–	Mature osteoclasts

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M-CSF	–	Macrophage colony stimulating factor
MMP-9	–	Matrix metalloproteinase 9
MSC	–	Mesenchymal stem cell
mTor	–	Mechanistic target of rapamycin
MUFA	–	Monounsaturated fatty acid
M-MuLV	–	Moloney murine leukaemia virus
$NaNO_2$	–	Sodium nitrite
NFATc1	–	Nuclear factor of activated T-cells, cytoplasmic 1
NF-κB	–	Nuclear factor-κB
NIK	–	NF-κB-inducing kinase
nNOS	–	Neuronal NOS
NO	–	Nitric oxide
NOS	–	NO synthase
OC	–	Osteoclast
OPG	–	Osteoprotegerin
OVX	–	Ovariectomized
PBMC	–	Peripheral blood mononuclear cell
PBS	–	Phosphate buffered saline
PCR	–	Polymerase chain reaction
PGE_2	–	Prostaglandin E_2
PGE_3	–	Prostaglandin E_3
PI3K	–	Phosphatidylinositol-3-kinase
PIP3	–	Phosphatidylinositol (3,4,5)-triphosphate
PKA	–	Protein kinase A
PlasDIC	–	Polarization-optical differential interference contrast
pNPP	–	para-Nitrophenylphosphate
PPAR	–	Peroxisome proliferator-activated receptor
PTH	–	Parathyroid Hormone
RANK	–	Receptor activator of nuclear factor κB
RANKL	–	Receptor activator of nuclear factor κB ligand
RNA	–	Ribonucleic acid
ROS	–	Reactive oxygen species

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Runx2	–	Runt-related transcription factor 2
S6K	–	Ribosomal protein S6 kinase beta-1
SFA	–	Saturated fatty acid
TAK1	–	TGF-β activated kinase 1
TGF-β	–	Transforming growth factor β
TLR4	–	Toll-like receptor 4
TNF-α	–	Tumor necrosis factor-α
TNFR	–	TNF-receptor
TRAF	–	TNF-receptor activating factor
TRAP	–	Tartrate resistant acid phosphatase
VC	–	Vehicle control
VNR	–	Vitronectin receptor (integrin $\alpha_v\beta_3$)

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CHAPTER 1

INTRODUCTION

1.1 Rationale for research

Osteoporosis is a bone disease characterized by an increase in the breakdown of bone by bone resorbing osteoclasts and a decrease in bone formation by osteoblasts. An estimated 200million people worldwide suffer from osteoporosis. [1] The current treatment involves drug therapies which can prove costly and may have side effects. The old saying goes "*prevention is better than cure*" and therefore affordable methods to effectively prevent and slow-down the occurrence of osteoporosis and osteoporotic fractures are being sought. Studies have shown that long chain polyunsaturated fatty acids (LCPUFAs) may have beneficial effects on bone health and that supplementation may improve the strength of bones. However, the mechanisms for the interactions between LCPUFAs and bone are not fully understood. *In vitro* studies on animal cell lines have helped shed some light on the possible mechanisms; however there are currently no published studies on the effects of LCPUFAs on a human osteoclast cell line.

1.2 Purpose of research

The aim of this study was to investigate the effects of arachidonic acid (AA), an ω-6 LCPUFA, and docosahexaenoic acid (DHA), an ω-3 LCPUFA, on osteoclastogenesis (osteoclast formation) and bone resorption in human CD14+ monocytes and to elucidate the mechanisms of action of these LCPUFAs in human osteoclasts.

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1.3 Method of investigation

The study was conducted *in vitro* on human CD14+ monocytes, isolated from peripheral blood. The cells were exposed to differentiation factors, receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony stimulating factor (M-CSF), to stimulate osteoclast formation. By using the CD14+ monocytes it allowed for experiments to be conducted on differentiating and mature osteoclasts as previously described by Agrawal *et al*. i.e. either the cells were exposed to AA or DHA from day 3 to test their effects on differentiating osteoclasts or the cells were exposed to the LCPUFAs from the onset of resorption (day 11-14) to test their effects on mature osteoclasts. [2] The effects were tested on a variety of factors that are characteristic of osteoclasts such as: osteoclastogenesis, TRAP activity, resorption, cell morphology, actin ring formation and the expression of prominent osteoclast receptors, proteins and genes.

1.4 Objectives

1. **Osteoclastogenesis and TRAP activity:**

   To elucidate the effects of AA and DHA on RANKL-mediated osteoclastogenesis by means of quantifying expression of tartrate resistant acid phosphatase (TRAP) in the conditioned growth medium and the cells themselves.

2. **Cell morphology:**

   To investigate the effects of AA and DHA on the cell morphology of differentiating CD14+ monocytes by polarization-optical differential interference contrast (PlasDIC).

3. **Resorption:**

   To determine the effects of AA and DHA on resorption by visualisation of the resorption pits on dentine slices.

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4. **Actin ring and receptor expression:**

   To investigate the effects of AA and DHA on the ability of osteoclasts to form actin rings, required for the structural integrity of osteoclasts, and express vitronectin receptor (VNR) and calcitonin receptor (CTR), both markers of mature osteoclasts, by means of immunofluorescent staining.

5. **Protein and gene expression:**

   To examine the effects of AA and DHA on protein marker expression and gene marker expression in osteoclasts by means of western blotting and polymerase chain reaction (PCR) respectively.

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CHAPTER 2

LITERATURE REVIEW

In this section the background knowledge considered to be most vital to the project will be addressed. It will begin with a brief description of bone, the different types of bone cells and receptor activator of nuclear factor κB (RANK) signalling which controls the formation of osteoclasts. The bone remodelling cycle, which continually replaces old bone with new bone, will be discussed in detail, and osteoporosis will be used as an example to explain the implications of a breakdown in this cycle. LCPUFAs will then be discussed and some benefits of LCPUFA consumption in early development, heart health, cancer and obesity will be examined briefly to show that LCPUFAs are not only beneficial for bone health but can improve health in general. Lastly, *in vitro* and *in vivo* research on the effects of LCPUFAs on bone and possible mechanisms of action of LCPUFAs on bone will be examined to gain a better understanding of where our current knowledge stands and which gaps this study hopes to fill.

2.1 Bone

Bone is an extremely dynamic tissue composed of inorganic and organic compounds. The inorganic component consists mostly of calcium phosphate crystals (mostly hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$)) while the organic component consists mostly of collagenous proteins. [3, 4] It consists of about 80% cortical bone, the dense and solid part of the bone that surrounds the marrow, and 20% trabecular bone, the honeycomb-like network of plates and rods in the marrow compartment. [3] There are four main types of bone cells; osteocytes which are mechanosensory cells embedded in the bone, osteoclasts which break down (resorb) bone, osteoblasts which build-up bone and bone lining cells which protect the bone surface.[3] Bone is remodelled continuously by a process known as the bone remodelling cycle, which is mediated by

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the coupling of bone resorbing osteoclasts and bone forming osteoblasts. [5] Derangement of the remodelling cycle is the major cause of bone diseases such as postmenopausal osteoporosis and hypocalcaemia, caused by excessive resorption; or osteopetrosis, caused by decreased resorption. [5] Therefore, the bone remodelling cycle is tightly controlled.

2.1.1 Bone cells

As stated earlier, bone remodelling is primarily controlled by two cell types, osteoclasts and osteoblasts. Osteoclasts are multinuclear cells that are derived from haematopoietic precursors of the monocyte/macrophage lineage, such as CD14+ monocytes in humans. [6] Promyeloid precursors will differentiate and fuse into osteoclasts when exposed to receptor activator of nuclear factor-κB ligand (RANKL) and macrophage colony stimulating factor (M-CSF) which are produced by osteoblasts. [7] M-CSF binding to c-Fms on pre-osteoclasts will stimulate the proliferation, differentiation and survival of promyeloid precursors while RANKL binding to RANK stimulates osteoclastogenesis and prevents osteoclast apoptosis. [8, 9] RANKL can either be membrane bound or enzymatically cleaved into a soluble form that maintains osteoclastogenic properties. [8] Several different cells such as T-cells, endothelial cells and osteoblasts can secrete M-CSF and RANKL. [8, 9] Osteoprotegerin (OPG), a soluble factor produced by a range of different cells including osteoblasts, inhibits osteoclastogenesis by binding to RANKL in place of the RANK receptor thereby neutralizing it. [7, 10] The balance between OPG, RANKL and M-CSF is important in regulating bone remodelling and maintaining the micro-structure of bone (Figure 2.1). [10, 11]

Osteoclasts are the sole bone resorbing cell in the body. [7] They are extremely large (reaching in excess of 300µm *in vitro*) terminally differentiated cells. [7] The cell usually contains 10-20 nuclei per cell, but has been found to contain over 100 nuclei per cell in patients with Paget's disease. [7, 12] The osteoclast has a polarized morphology, with a

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functional secretory zone facing away from the bone, and a resorptive surface that faces the bone. [5, 7, 12]

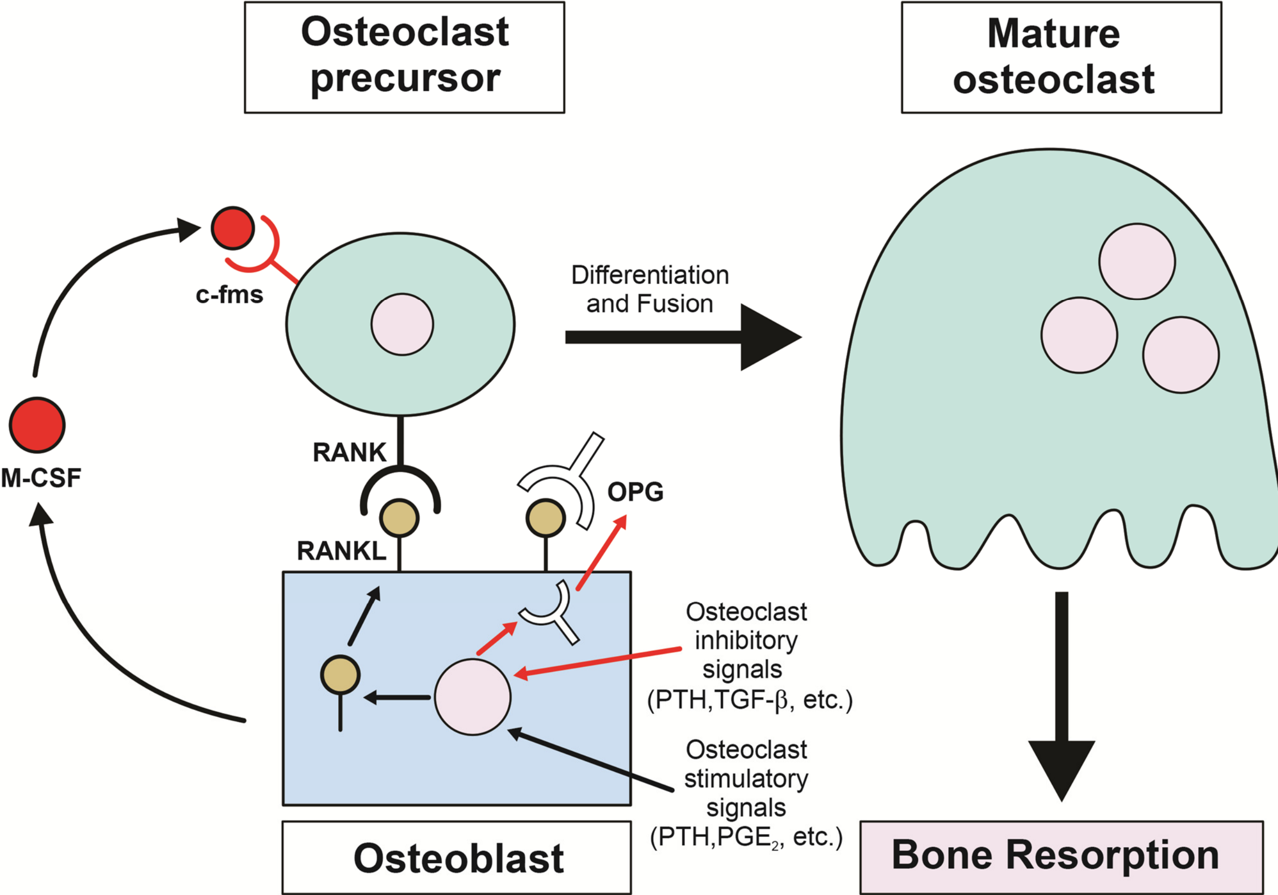

Figure 2.1: The control of osteoclastogenesis by OPG, M-CSF and RANKL. In response to osteoclast stimulatory signals (black arrows), osteoblasts will produce RANKL and M-CSF. Both bind to osteoclast precursors to stimulate osteoclast differentiation and resorption. In response to osteoclast inhibitory signals (red arrows), osteoblasts will produce OPG which will act as a decoy receptor for RANKL and prevent osteoclast differentiation and resorption. Imaged created with information from Yasuda *et al*. (1998). [10]

At sites of bone contact, the osteoclast forms F-actin rings and a sealing zone between the bone and the cell membrane which is mediated by integrin $\alpha_v\beta_3$, also known as vitronectin receptor (VNR). [5, 12] Inside the sealing zone, the resorptive surface forms a ruffled border which is the resorbing organelle of the osteoclast (Figure 2.2). [5, 12] At this stage there are several unique biochemical features of the osteoclast which are functionally important and can be used for identification such as the presence of receptors VNR and calcitonin receptor (CTR) and resorbing enzymes matrix metalloproteinase-9 (MMP-9), cathepsin K and tartrate-resistant acid phosphatase

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(TRAP) which resorb the organic component of bone. [12] The most commonly used marker for osteoclastogenesis is TRAP, which is an enzyme that appears early on in osteoclastogenesis but increases in expression throughout the maturation process. [12] The exact role of TRAP is unknown but it is believed to be involved in cell growth and differentiation as well as the formation of reactive oxygen species needed for resorption. [13] Hydrochloric acid is pumped into the resorption pit to resorb the inorganic component of bone and because an acidic environment is required for the optimal functioning of the resorptive enzymes. [14]

Figure 2.2: The multinucleated osteoclast adhering to bone. A sealing zone is formed that is acidified by hydrogen ions (H^+), through H^+ ATPase. VNR ($\alpha_v\beta_3$) assists in the formation of the sealing zone. Cathepsin K (CTSK) and MMP-9 are released into the resorption pit to resorb the organic matrix, while acidification takes place to break down the mineral content of bone. Degradation products are endocytosed with CTSK where they fuse with vesicles containing TRAP. The transcytotic vesicle is then released with its products through the functional secretory zone. Image created with information from Ross *et al*. (2006) and Takahashi *et al*. (2014). [15, 16]

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Osteoblasts have the opposite role of osteoclasts, as they are responsible for laying down new bone after osteoclasts have resorbed a specific area. They are mononuclear cells that have a cuboidal morphology and are much smaller than osteoclasts, about 15-30µm. [12, 17] During bone remodelling, osteoblasts produce factors such as OPG, M-CSF and RANKL to control osteoclast formation and resorption in response to certain signals. [10] Osteoblasts originate from mesenchymal stem cells (MSCs), which are multipotent and can differentiate into numerous different cell lines such as osteoblasts, adipocytes, chondroblasts and myoblasts depending on the secretion of different master transcription factors. [12, 18] Core binding factor alpha (Cbfa)-1 (also known as Runx2) is a transcription factor that can initiate osteoblast formation and its expression is the earliest identified event of osteoblastogenesis. [12, 19] Cbfa-1 is known to stimulate the formation of osteoblasts from MSCs by up-regulating the expression of osteoblastic genes such as osteopontin, osteocalcin and osterix. [12, 18] Interestingly, an over-expression of Cbfa-1 in pre-adipocytes can cause them to change phenotype and become osteoblasts, indicating these cells may not be terminally differentiated. [12] Studies have shown that peroxisome proliferator-activated receptor (PPAR)-γ activity may inhibit the formation of bone by directing MSCs from osteoblast differentiation to adipocytic differentiation. [20] Besides PPAR-γ, there are two other identified PPARs: PPAR-α and PPAR-β/δ; both of which are expressed by osteoblasts and can promote osteoblast differentiation. [21]

The majority of osteoblasts will undergo apoptosis while the remaining osteoblasts will be embedded in the bone where they will become osteocytes the most abundant bone cell (making up 90-95% of bone cells) or remain on the surface where they will become bone lining cells. [12, 22] Osteocytes play a role in the cell-to-cell signalling between osteoclasts and osteoblasts which is needed to control bone remodelling (See 2.1.3). [12, 22] While embedded in the bone, osteocytes are connected to each other by long slim cellular processes, known as canaliculi, which allow communication and exchange of intercellular material between osteocytes. [23]

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2.1.2 Receptor activator of nuclear factor κB (RANK) signalling

As RANKL is the only known ligand of RANK, RANK and RANKL are crucial in bone metabolism. The binding of RANKL to RANK triggers an intricate signalling cascade that leads to commitment of pre-osteoclasts to form osteoclasts. [24] Although not fully understood, much of the pathway has been described in efforts to understand the osteoclast specific signalling pathway (Figure 2.3).

As RANK has no intrinsic kinase activity, like other tumour-necrosis factor (TNF) receptor family members, it cannot phosphorylate or activate downstream signalling molecules. [24] Therefore it relies on TNF-receptor activating factors (TRAFs), in particular TRAFs 1, 2, 3, 5 and 6, to act as adaptor molecules to recruit protein kinases. [25] In the absence of RANKL, TRAF-2 and -3 form a complex that degrades nuclear factor κB (NF-κB) inducing kinase (NIK). [26] NIK is a kinase that degrades inhibitory kappa kinases (IKKs) freeing NF-κB to enter the nucleus and therefore the degradation of NIK leads to an inhibition of osteoclast formation. [25] NF-κB deficient mice have been shown to be osteopetrotic and develop defective formation of the growth plate and dwarfism indicating NF-κB plays a vital role in osteoclast formation. [26, 27] RANK-RANKL binding results in the degradation of TRAF 3 which allows NIK to accumulate and results in osteoclast formation. [25]

TRAF-6 deficient mice have been shown to exhibit severe osteopetrosis showing that it is crucial to osteoclast formation and function. [28] TRAF-6, by interacting with transforming growth factor (TGF) -β activated kinase 1 (TAK1), can activate NF-κB (through NIK) and c-Fos (through c-Jun N-terminal kinase (JNK1)), both of which activate nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1). [22, 26-28] NFATc1 is known as the master regulator of osteoclastogenesis and signals the formation of osteoclasts and osteoclast specific genes. [29] Dendritic cell-specific trans-membrane protein (DC-STAMP) is a notable gene that is highly expressed in the early stages after NFATc1 activation and plays a role in cell-to-cell fusion during osteoclast formation. [30]

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Figure 2.3: RANK signalling. Binding of RANKL to RANK commits pre-osteoclast to form osteoclasts. The TRAF-6/TAK1 complex activates the MAPK pathways, JNK1 (activating c-Fos and NFATc1), p38 and ERK1 to stimulate osteoclast formation, growth and resorption. TRAF6/TAK1 complex also activates the NF-κB pathway triggering the activation of NFATc1 which activates several osteoclast specific genes. The TRAF6/c-Src complex causes actin reorganization as well as activating PI3K which stimulates cell growth and survival. Binding of M-CSF to its receptor (c-Fms) stimulates the expression of the RANK gene. Image created with information from Aeschilmann *et al.* (2004) and Wada *et al*. (2006). [24, 29]

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TRAF-6/TAK1 triggers the activation of other mitogen activated protein kinase (MAPK) family members such as JNK1, p38 and extracellular signal-regulated kinase 1 (ERK1) which mediate osteoclastic resorption and survival respectively, although the exact signalling pathway is not fully understood. [24, 25, 29] ERK1 may play a role in activating c-Fos expression in osteoclasts as it has been shown to do so in intrauterine tissue. [31] MAPKs are known to play a role in activating carbonic anhydrases (CAs), such as CA2, which plays a role in the formation of the acidic environment required for resorption. [32]

TRAF-6, by interacting with tyrosine-protein kinase CSK (c-Src), can activate actin reorganization, as well as the activation of phosphatidylinositol-3-kinase (PI3K) which, through a cascade of enzymes, results in increased cell survival and growth. [29] PI3K will activate phosphatidylinositol (3,4,5)-triphosphate (PIP3) which will trigger down-stream proteins (Akt, mTOR, S6K, etc.). The binding of M-CSF to its receptor, c-Fms, can also trigger actin reorganization and increased cell survival and growth through c-Src and PI3K (similar pathway as TRAF6/c-Src). [29] M-CSF furthermore triggers an increase in the expression of the RANK gene thereby increasing the availability of the receptor for RANKL. [24]

2.1.3 The bone remodelling cycle

During childhood and adolescence, when growth is still occurring, a process known as bone modelling occurs, where the rate of bone production by osteoblasts exceeds the rate of bone resorption by osteoclasts. Consequently the bones can grow in length and width. At this stage resorption and formation are not tightly coupled. [3] Bone modelling is less present in adulthood, where bone remodelling, a continuous process of breaking down old bone and replacing it with new bone in order to replace old bone, repair fractures and prevent the accumulation of micro-damage, becomes more prevalent. [3] Mature bone remodels with an estimated turnover rate of 10% per year. [33] There are 5 major steps in the remodelling cycle: activation, resorption, reversal, formation and termination (Figure 2.4). [14]

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A - Activation
RANKL
MCSF
B - Resorption
E - Termination
Parathyroid Hormone (PTH)
Sclerostin
C - Reversal
EphB4
EphrinB2
D - Formation
TGFβ
Bone lining cells
Osteoclast
Microfracture
Osteocyte
Osteoblast
EphrinB2
EphB4

Figure 2.4: The bone remodelling cycle. A) Activation is most likely triggered by microfractures or strain on the bone which are detected by osteocytes and/or bone lining cells which attract osteoclast precursors by secreting RANKL and M-CSF. **B)** Osteoclast precursors mature into bone resorbing osteoclasts which resorb the area until signals by osteocytes and osteoblasts halt resorption. **C)** Membrane proteins EphrinB2 on osteoclasts and EphB4 on osteoblasts and factors such as TGF-β are believed to be involved in the communication between the two cells leading to the differentiation of osteoblasts and activation of bone formation **D)** Osteoblasts lay down the osteoid which will later be mineralized. Sclerostin, released by osteocytes is believed to be responsible for the termination of the remodelling cycle. **E)** The majority of osteoblasts undergo apoptosis while a few will remain in the bone where they will become osteocytes or bone lining cells. PTH is known to stimulate resorption, reversal and formation, but inhibit termination. Image created with information from Henriksen *et al*. (2009). [14]

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Activation occurs when a microfracture appears in the bone and disrupts either the bone lining cells or the canaliculi between osteocytes embedded in the bone.[14] Strain levels on the bone have also been shown to activate osteocytes and trigger remodelling. [34] In response to strain or a microfracture, the osteocytes or the bone lining cells will stimulate osteoclast precursors and attract them to the area. [22] This is most likely through the release of RANKL and M-CSF that cause the maturation and differentiation of osteoclast precursors. [14] The signalling pathway however, is not fully understood.

Once activated, osteoclasts will migrate to the microfracture site and resorption will occur. The main function of osteoclasts is the resorption of both the inorganic and organic structures of bone. [5, 35] The resorption process requires many cellular activities, most notably: the migration of osteoclasts to the resorption site; the attachment of the osteoclast to the resorption site; the release of degrading factors to break down the bone; the removal of the degraded products; and the apoptosis of the osteoclast or its return to a non-resorption stage. [5] After osteoclasts attach to the bone and form a sealing zone between the cell membrane and the bone, hydrochloric acid, released through an H^+ ATPase and charge-coupled Cl^- channel, is released to acidify the resorption microenvironment and dissolves the inorganic component of bone (Figure 2.2). [5, 12] Carbonic anhydrases, particularly CA2, catalyses the formation of hydrogen ions (H^+). The lysosomal enzymes MMP-9 and cathepsin K, which require an acidic environment to function optimally, are released to degrade the organic matrix. [12] The degradation products together with cathepsin K are then endocytosed into the osteoclast where they fuse with vesicles containing TRAP (Figure 2.2). [5, 7, 36] Cathepsin K will cleave TRAP into an activated form. [36] TRAP is also released into the resorption pit where it aids in the formation of reactive oxygen species needed to break down bone. [13] The contents of the vesicle are released into the extracellular space. This allows for the identification of osteoclast markers in the conditioned medium, *in vitro*. The whole process of resorption by osteoclasts takes 2-4 weeks to complete. [3]

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Once osteoclastic resorption is completed, the osteoclasts will undergo apoptosis. [37] The exact signals that determine the completion of resorption are unknown, though they are believed to be produced by osteocytes, possibly with assistance from osteoblasts. [37] Mononuclear cells of osteoblastic lineage migrate to the area and are believed to be responsible for "cleaning up" debris left behind by osteoclasts and further degrading any remaining collagen in preparation for bone formation. [14, 37, 38] Studies have also shown that the expression of membrane proteins EphrinB2 by osteoclasts and EphB4 by osteoblasts mediates communication between these two cell types, and this could potentially initiate osteoblast differentiation during the reversal phase of bone remodelling. [14]

Now that the bone has been resorbed and the area has been cleared, osteoblastic bone formation can occur. Bone formation is the longest phase of the remodelling cycle, taking 3-6 months to complete, and it involves replacing the resorbed area with new osteoid (unmineralized bone) that will eventually be mineralized. [3, 38] The mechanisms involved in attracting osteoblasts to the resorption area are not fully understood. However, there are many factors that are released from the bone during osteoclastic resorption (transforming growth factor (TGF)-β, bone morphogenetic proteins (BMPs), etc.) that have anabolic effects on osteoblasts. [14, 38] Osteoclasts also play a role by releasing factors such as TRAP, which are also known to have stimulatory effects on osteoblasts. [14] As osteoblasts replace the bone, the more mature osteoblasts become entrapped in the bone where they will become osteocytes. [38] After formation is complete, 50-70% of osteoblasts will undergo apoptosis with the remaining osteoblasts becoming osteocytes or bone lining cells. [3] At the conclusion of the remodelling cycle, the bone will be replaced but the total amount of bone will remain unchanged. Only in pathological conditions such as osteoporosis (increased resorption and decreased formation) and osteopetrosis (increased formation and decreased resorption) will the total amount of bone change.

The termination of bone remodelling is the last major phase of the cycle. Studies have shown that osteocytes can regulate osteoblasts activity through direct contact or

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through gap junctions. [39, 40] Furthermore, osteocytes secrete sclerostin which has been shown to inhibit osteoblast formation by preventing the activation of osteoblast inducing signals. [14, 38] The trigger for the secretion of sclerostin still remains unclear. The parathyroid hormone (PTH) plays an important role in regulating bone remodelling as it can trigger resorption, reversal and formation. [14] PTH can also inhibit sclerostin. [14] PTH is secreted in response to low levels of calcium. At high concentrations it is known to stimulate bone resorption, however, low, intermittent doses of PTH have been shown to increase bone mass and structure. [41]

2.1.4 Osteoporosis - a breakdown in the remodelling cycle

Osteoporosis is the result of a decreased bone mass and interference in the micro structure of bone. [42] This can increase the risk of bone fractures which can affect the quality of life of the individual suffering from this condition. One in two women and one in five men over the age of 50 may suffer a fracture as a result of osteoporosis and the resultant estimated annual cost to health services in Europe is about $30 billion. [42] In the US, two million osteoporotic fractures occur annually with an estimated cost of $17 billion: this number is expected to increase by 50% by 2025. [43] Osteoporosis generally goes undiagnosed until fragility fractures occur, at which time treatment may prove costly and burdensome. [44]

2.1.4.1 Pathogenesis

Fragility of the skeleton can be caused by three factors: 1. an inadequately produced skeleton during growth, 2. excessive resorption by osteoclasts, 3. an inability of osteoblasts to adequately replace bone that has been lost during remodelling. [45] These factors can all affect an individual's ability to maintain peak bone mass (the maximum bone mass achieved in life). [46] Peak bone mass is generally achieved between ages 30-40, and loss of bone mass can lead to fragility fractures. [46] The adult skeleton is the result of peak bone mass, achieved early in life, and the maintenance of the architecture and bone density of skeleton, achieved later in life. [47] Genetics, hormones

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and several other regulatory and pathogenetic mechanisms can affect peak bone mass.

There are 80 known genetic loci that influence bone mineral density (BMD). [46] Many of these loci are involved in RANK/RANKL/OPG signalling and the differentiation of MSCs. However, variations in these genes do not contribute significantly to variations in BMD. Furthermore, there have been no association studies between genome and loss of BMD, and therefore the influence of these genes on the rate of bone loss still remains unclear. [46]

Although the mechanism of action is poorly understood, sex steroids are known to play a role in bone health. It has been suggested that oestrogen aids in the recognition of the strain threshold, which is needed for bone cells to adaptively respond to strain levels, leading to normal bone remodelling. [48] Oestrogen has also been shown to prevent osteocyte apoptosis, maintain osteocyte numbers, promote osteoclast apoptosis and prevent bone resorption. [48] The rate of bone remodelling has been shown to increase at menopause (or after castration in men). [49] Both osteoclast and osteoblast activity are increased in post-menopausal women. [45] However, as osteoclastic resorption takes place at a faster rate than osteoblastic bone formation, this will undoubtedly result in a loss of BMD.

Studies have shown that between menopause and 75 years of age, women lose about 22% of their total bone mineral content. [50] However, only 7.75% is attributed to oestrogen deficiency, while 13.3% is the result of aging. [46] During aging, the balance between bone resorption and formation increases in favour of resorption. [46] Oxidative stress, the primary cause of several degenerative diseases associated with aging, is the suspected culprit of age associated osteoporosis. [51] Reactive oxygen species (ROS) have been the suspected cause of decreases in bone formation as they are known to stimulate osteoblast apoptosis. [52]

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Chronic inflammatory conditions have commonly been linked with the loss of bone. [53] This may be due to the increase in pro-inflammatory cytokines such as TNF-α and certain interleukins (IL-1, IL-6, IL-11, IL-15 and IL-17). [54] RANK and RANKL are mediators of these pro-inflammatory cytokines, and therefore this represents a mechanism through which pro-inflammatory cytokines can increase osteoclast activity and bone loss; ultimately leading to bone diseases such as osteoporosis. [54] IL-4, an anti-inflammatory cytokine, is known to inhibit IL-17 as well as RANKL and could potentially reduce osteoclast activity and bone loss. [55, 56] The balance between pro-inflammatory and anti-inflammatory cytokines may be crucial in the pathogenesis of osteoporosis.

Differences in ethnicity are also known to contribute to peak bone mass. African-Americans are known to have higher BMDs than Caucasians, while Asian-Americans have lower BMDs. [46] Environmental factors such as poor growth, malnutrition, physical inactivity, etc. also play a role. High levels of glucocorticoids, whether endogenous or pharmacological, have been shown to decrease bone mass and lead to osteoporosis. [49] Several more mechanisms can be attributed to an increase in bone fragility. A low peak bone mass may contribute to the development of osteoporosis later in life. However, increased age and a decrease in sex steroids, coupled with a tendency to fall are the critical indicators of an increased risk of fragility fractures.

2.1.4.2 Treatment

Oestrogen deficiency is known to play a role in bone loss in post-menopausal women, and oestrogen levels have also been shown to play a role in bone loss in elderly men, putting these patients at higher risk of fractures. [42] While oestrogen replacement therapy has been used effectively to combat post-menopausal conditions including osteoporosis, the pro-mitotic effects of oestrogen mean that replacement therapy may have side effects, such as increasing the risk of developing breast and ovarian cancers.[57] In addition to this, due in part to the increased survival of chronically ill children who may be exposed to treatment that is toxic to the skeleton, osteoporosis is also becoming an increasing problem among children and young adults. [58, 59] Besides

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the obvious side effects to oestrogen replacement therapy, it would not prove useful to children and young adults where oestrogen levels are normal. Therefore it does not present an ideal solution to combating osteoporosis.

Apart from oestrogen replacement therapy, there are other pharmaceutical interventions that are used in the treatment of osteoporosis. Bisphosphonates have been the most commonly prescribed agents as they have been shown to reduce vertebral as well as non-vertebral fractures. [42, 60] Bisphosphonates are generally taken orally while fasting, and with a full glass of water; the individual must refrain from eating any food for 30-60 minutes after ingesting the bisphosphonates. [42] Apart from gastrointestinal problems that can occur if the subject is not compliant to the dosing regimen, long-term use of bisphosphonates may also cause other side effects such as, osteonecrosis of the jaw, and femoral fractures. [60] However, the adverse effects related to the use of bisphosphonates are rare in comparison to the benefit of reducing the risk of fractures. [60] Strontium ranelate and Raloxifene have also been shown to reduce non-vertebral and vertebral fractures, however side effects include diarrhoea, headaches, hot flashes and leg cramps. [42]

Another major pharmaceutical treatment for osteoporosis is the use of the anabolic agent teriparatide (recombinant 1-34 parathyroid hormone [PTH]) as well as preotact (the full 1-84 PTH), however teriparatide is currently the only approved anabolic therapy for osteoporosis in the United States. [42, 61] As seen in Figure 2.4, PTH plays a crucial role in bone remodelling. However, these medications do not reduce the risk of hip fractures and are more costly than the other therapies. Therefore they are reserved for patients who are unresponsive to the other treatments. [42] Both teriparatide and preotact can stimulate the formation of bone by affecting bone modelling and remodelling. [60] The most significant site of increasing bone mineral density is usually in the spine. [61] Besides the high cost of using these treatments, studies have shown that high levels of teriparatide can increase the risk of osteocarcinoma in rats. [62-65] Dizziness, hypercalcemia, leg cramps and hypercalciuria have also been reported as side effects of teriparatide. [61]

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Denosumab is a recently developed prescription medication that has been approved for the treatment of post-menopausal osteoporosis by the *US Food and Drug Administration* under the trade name, Prolia®. Denosumab is a human monoclonal antibody that can inhibit RANKL activity by preventing it from binding to RANK, mimicking the action of OPG (Figure 2.1). [66, 67] This dosing regimen is relatively non-invasive as, generally, only one 60mg subcutaneous injection is admitted every 6 months. [66] Studies have shown that denosumab can increase BMD and bone strength and reduce the risk of fractures in post-menopausal women with osteoporosis with few, mild adverse effects seen with long term use. [67-71] However, a study has shown that denosumab may increase the occurrence of eczema and other allergic skin conditions, as RANKL may play a role in the normal immune response. [71] In normal conditions, RANKL is highly expressed by immune cells such as T-cells. [8]

2.2 Long chain polyunsaturated fatty acids (LCPUFAs)

Over the past few years, research has focused on non-pharmaceutical therapies in the treatment of osteoporosis in order to reduce drug dependency, harmful side effects and costly hospitalisation. [72, 73] Increasing dietary intake of calcium and vitamin D, as well as increasing exercise and discouraging smoking and alcohol abuse have all been recommended to help increase health and prevent osteoporosis. [42] There is an increasing body of evidence that suggest that LCPUFA have a beneficial effect on bone health. [73] In Inuit communities and regions such as Japan where there is a high dietary consumption of fish, high in ω-3 LCPUFAs, the occurrence of osteoporosis is very low. [21] The use of LCPUFAs is affordable and generally has no side effects and may be used as a preventative measure in the fight against osteoporosis. Although the beneficial effects of LCPUFAs on bone health have been studied extensively, the mechanisms involved have not been fully elucidated.

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2.2.1 Types of fatty acids

Fatty acids can be divided into two groups: saturated and unsaturated fatty acids. Saturated fatty acids (SFAs) are generally considered "bad fats" as they increase low density lipoprotein (LDL) cholesterol (also known as bad cholesterol). [74, 75] Unsaturated fatty acids are considered "good fats" as they have been shown to lower cholesterol, blood pressure and triacyglycerol levels. [74, 75] Fatty acids consist of a carboxyl group (-COOH) on one end, followed by an aliphatic tail (hydrocarbon chain) and a methyl group (-CH_3) on the other end. [21] Saturated fatty acids contain no double bonds in the aliphatic tail while unsaturated fatty acids will contain at least one double bond in the aliphatic tail. Unsaturated fatty acids can either be monounsaturated (MUFAs) (only one double bond in the aliphatic tail) or polyunsaturated (PUFAs) (at least two double bonds in the aliphatic tail). They can further be grouped by different geometric configurations; *cis*- and *trans*- configurations. [76] These geometrical isomerisms have implications on the physical attributes and functionality of the fatty acids as, where *cis*-fatty acids will have a bend in the chain, *trans*-fatty acids will have a straight chain and therefore act similarly to saturated fatty acids (See Figure 2.5). [76] *Trans*-fats typically are not found in nature, with the exception of some meat products, and are usually produced industrially. [77]

PUFAs can further be grouped by the length of their aliphatic tails. LCPUFAs are fatty acids with a minimum of 18 carbons and two double bonds in their aliphatic tails. [21] Most LCPUFAs will be in *cis*- configuration. LCPUFAs can be classified into two principal families, namely: ω-3 LCPUFAs and ω-6 LCPUFAs. This nomenclature refers to the position of the first double bond from the methyl end of the fatty acid chain: the first double bond is located on carbon 3 in ω-3 LCPUFAs and on carbon 6 in ω-6 LCPUFAs. [21] Omega-3 LCPUFAs, which are known to be anti-inflammatory, are derived from alpha-linolenic acid (ALA) while the pro-inflammatory ω-6 LCPUFAs are derived from linoleic acid (LA). [11, 21, 72, 78] As humans cannot synthesize double bonds after carbon 9 from the carboxyl end, both ALA and LA are essential fatty acids and must be supplied in the diet. [11, 21]

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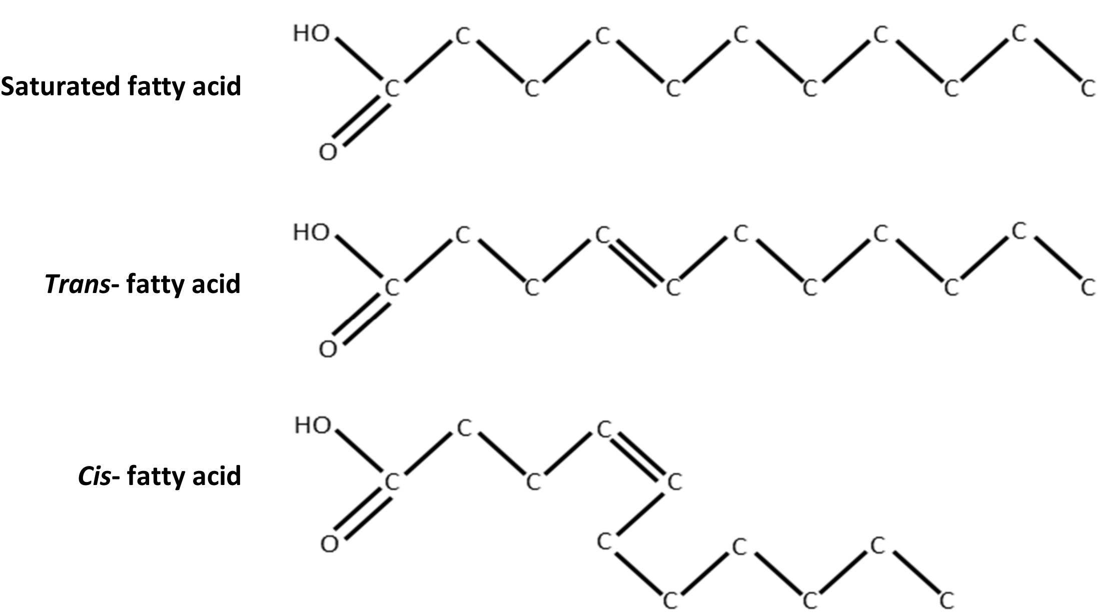

Figure 2.5: Comparison of the structures of saturated, *trans*-, and *cis*- fatty acids. *Trans*-fatty acids have a straight configuration similar to saturated fatty acids and will therefore function in much the same way. *Cis*-fatty acids have a clear bend in the chain leading to differences in functionality of these fatty acids. Adapted from Abedon. [77]

A series of shared enzymes can metabolize LA and ALA into several different metabolites. The most common metabolites of LA and ALA in the human body are AA and DHA respectively (Figure 2.6). [79] Metabolism of ω-6 LCPUFAs by these enzymes generally takes precedence over the metabolism of ω-3 LCPUFAs which occurs at a slower rate. [80] In diets high in ω-6 LCPUFAs, the conversion of ALA to EPA and DHA is insufficient and these LCPUFAs must be supplemented from the diet. [81] LCPUFAs can be further metabolized by cyclooxygenases (COX), lipoxygenases (LOX) and reactive oxygen species (ROS) to form prostaglandins, leukotrines and other metabolites. [73] These metabolites have autocrine and paracrine functionalities within the body. LCPUFAs have a wide range of health benefits including improving childhood development, anti-aging, anti-obesity, anti-cancer and cardio-protective effects. [75]

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ω-3 LCPUFAs

18:3 (9, 12, 15)
alpha-linolenic (ALA)

18:4 (6, 9, 12, 15)
stearidonic acid

20:4 (8, 11, 14, 17)

3-series Prostaglandins, Throboxanes & Lipoxins (COX)

Leukotrienes, Lipoxins (LOX)

Isoprostanes (ROS)

20:5 (5, 8, 11, 14, 17)
eicosapentaenoic (EPA)

22:5 (7, 10, 13, 16, 19)
docosapentaenoic

Tri-hydroxy derivatives

13R-HDHA, 17R-HDHA (COX)

LOX

D-series Resolvin, (Neuro) protectin, Other S-enantiomer di-hydroxy derivatives (LOX)

22:6 (4, 7, 10, 13, 16, 19)
docosahexaenoic (DHA)

COOH
CH_3

6-desaturase enzyme

elongase

5-desaturase

elongase

ω-6 LCPUFAs

18:2 (9, 12)
linoleic (LA)

18:3 (6, 9, 12)
gamma-linolenic (GLA)

20:3 (8, 11, 14)
dihomo-gamma-linolenic (DGLA)

20:4 (5, 8, 11, 14)
arachidonic (AA)

COOH
CH_3

2-series Prostaglandins, Throboxanes & Lipoxins (COX)

Leukotrienes, Lipoxins (LOX)

Isoprostanes (ROS)

Figure 2.6: Metabolism of ALA and LA by shared enzymes. LCPUFAs are commonly written in the format C:D (n), with C referring to the number of carbons, D the number of double bonds and (n) the position of the double bond from the carboxyl end. The two LCPUFAs to be used in this study, docosahexaenoic acid (DHA, ω-3) and arachidonic acid (AA, ω-6), are highlighted and their structures are shown. These FAs can be further metabolized by COX, LOX and ROS to form the metabolites as shown in the diagram. Although not shown, all the double bonds in this figure are in *cis-* configuration. Image created with information from Kurlak (1999) and Kruger *et al*. (2010).[73, 79]

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2.2.2 Benefits of long chain polyunsaturated fatty acids

2.2.2.1 Long chain polyunsaturated fatty acids in early development

Lipids in breast-milk are the main source of energy required for healthy growth in the neonates. [82] LCPUFAs, particularly AA and DHA have been shown to accumulate in large quantities in the developing brain. [82, 83] AA is believed to play a role in cell signalling, and is also a known precursor of prostaglandins and leukotrienes that have functions in several physiological processes. [82] DHA is the most abundant LCPUFA in the brain and is a component of brain cell membranes and the retina, but is also known to play a role in neurogenesis, neurotransmission, and protecting the brain and retina against oxidative stress. [82] DHA, AA and other LCPUFAs are made available to infants through breast-milk and formula and studies have shown a positive correlation between visual acuity and mental development in preterm infants supplemented with LCPUFAs. [83-87] Harvey *et al*. have also shown a positive association between maternal LCPUFAs levels during pregnancy and bone health in offspring at 4 years. [88] These studies suggest that LCPUFAs are essential in early childhood development and that LCPUFA supplementation can improve development, especially in preterm infants.

2.2.2.2 Long chain polyunsaturated fatty acids and heart health

For many years, the intake of LCPUFAs, particularly ω-3 LCPUFAs, have been recommended to reduce the risk of cardiovascular diseases. Studies have shown that fish oil which is rich in ω-3 LCPUFAs may have cardioprotective effects due to the fact that they lower triacylglycerols and have antihypertensive, anti-thrombotic, and anti-inflammatory effects. [89] Observational studies have shown that Greenland's Inuit community have a low risk of death from coronary heart disease which has been attributed to their diet that is high in ω-3 LCPUFAs. [90]

Many studies have also shown that Japanese men, who have diets high in ω-3 LCPUFAs, have lower instances of atherosclerosis than Caucasian males in the US. [91]

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When the incidence of atherosclerosis was compared between Japanese men living in Japan and Japanese men living in the US, it was found that Japanese men in Japan had lower levels of atherosclerosis than their counterparts living in the US; also noted was that the Japanese men living in the US had similar levels of atherosclerosis to Caucasian men in the US. [91] The Japanese diet typically contains 8-15 times more ω-3 LCPUFAs than that of people in other developed countries. [90] This ω-3 LCPUFA rich diet is believed to be the cause of the low levels of atherosclerosis observed in Japan. Further studies have shown that ω-3 LCPUFA, but not ω-6 LCPUFA, supplementation in patients already suffering from atherosclerosis has a stabilizing effect on the atherosclerotic plaque, possibly explaining the decrease in cardio-vascular events associated with an increased ω-3 LCPUFA intake. [92]

Several other studies have shown that ω-3 LCPUFAs supplementation has been attributed to lower instances of heart failure, arrhythmias and coronary artery disease, lower heart rates and reduced blood pressure as well as improved myocardial function. [90, 91, 93] The beneficial effects of ω-3 LCPUFAs on cardiovascular health still remain somewhat controversial due to the possible rise in the risk of arrhythmia and the fact that recent studies seem to show a lesser effect than earlier studies. [90, 94] However, it has been suggested that this may be due to the fact that the knowledge of the beneficial effects of ω-3 LCPUFAs on heart health are now widely known and this may have increased background intake of ω-3 LCPUFA supplements. [94] Therefore intervention studies may show no perceivable effect on ω-3 LCPUFA supplementation because the subjects have already been increasing their intake of ω-3 LCPUFAs for years.

Omega-6 LCPUFAs have been shown to reduce coronary heart disease and cardiovascular disease as well. [80] However, due to their pro-inflammatory effects, individuals with inflammatory conditions may be advised to lower ω-6 LCPUFA intake. [80] Due to the fact that ω-6 LCPUFA metabolism takes precedence over ω-3 LCPUFAs, increasing ω-6 LCPUFA intake can have the effect of lowering ω-3 LCPUFA

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metabolites in the body. Therefore, finding an optimum ratio of ω-6/ω-3 LCPUFA consumption has many conceptual and biological limitations. [80] Nevertheless, the *Dietary Reference Intakes for Energy, Carbohydrate, Fiber, Fat, Fatty Acids, Cholesterol, Protein, and Amino Acids (Macronutrients)* recommends a ratio of ω-6/ω-3 of 5:1 to 10:1 for adults. [95] Furthermore, the *American Heart Association* has recommended the consumption of at least two fish meals per week and 1g of ω-3 LCPUFA supplementation per day for individuals who have suffered a myocardial infarction. [90] The recommended intake of ω-6 LCPUFAs to reduce the risk of coronary heart disease is between 3-10% of total energy intake. [80]

2.2.2.3 Long chain polyunsaturated fatty acids and cancer

Diet is believed to be a contributory factor in 30-60% of cancers worldwide. [96] Several publications have shown that ω-3 and ω-6 LCPUFAs have cytotoxic effects on some cancers as well as synergistic effects with cytotoxic drugs, *in vitro* and *in vivo* in animals. [97-104] The modulation of angiogenesis by ω-3 LCPUFAs, which are known to decrease angiogenesis, is a proposed mechanism to their cytotoxic effects on cancer cells. [105] Other possible mechanisms include the regulation of gene expression, the impact on oxidative stress, altering the metabolism of hormones, and modifying cell-signalling pathways. [106] Despite these positive results in cell cultures and animal studies, very few human trials have been done with anti-cancer drugs in conjunction with LCPUFAs. [104, 107, 108] These few studies have shown some very promising results. Synergistic effects between DHA and the chemotherapeutic agent placitaxel in patients with refractory solid tumours were observed by Wolff *et al*. [107] Similar effects were seen with the addition of gamma-linolenic acid (GLA) and the chemotherapeutic agent tamoxifen in patients with endocrine sensitive breast cancer. [108] These results show that both ω-3 and ω-6 LCPUFAs can have synergistic effects with chemotherapeutic agents in humans. The combination of LCPUFAs with these drugs is of importance as it could allow for the use of lower doses of chemotherapeutics with LCPUFAs to achieve the same therapeutic results as higher doses of the anti-cancer drugs. This could reduce some of the side effects of chemotherapy.

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2.2.2.4 Long chain polyunsaturated fatty acids and obesity

Despite the *World Health Organization* claiming that obesity is one of the 10 most preventable health risks, 300 000 Americans still die of obesity related complications annually. [109] An estimated 1.6 billion people worldwide are overweight and at least 400 million are obese. [110] While genetics may play a role, obesity is generally considered as a lifestyle disease. The consumption of dietary fat has long been assumed to be an associated risk factor in obesity. [111, 112] However, studies have shown that in the US, the percentage of calories obtained from dietary fats has decreased while the rate of obesity has increased. [113] These results suggest that the type of dietary fat consumed is far more important than the amount. As our diets have evolved, we have moved from a diet of wild animals, containing large amounts of LCPUFAs (especially ω-3 LCPUFAs), to a diet of farm animals containing more *trans*- and saturated fats and almost devoid of ω-3 LCPUFAs. [109] This change in our dieting habits, coupled with a decrease in physical exercise, could be a fundamental cause in the increase in obesity that is present in our modern society.

LCPUFAs, unlike saturated and *trans*- fatty acids, have been shown to have anti-obesity effects in animal studies. [114] Studies have shown a protective effect on fat gain as well as obesity reduction when ω-3 LCPUFAs have been incorporated into a high fat diet in mice. [114] Human studies are not as conclusive as there is little data from well controlled studies available. [114] In a study of six healthy adults, Couet *et al*. where able to show that when fat from the diet was replaced with fish oil rich in ω-3 LCPUFAs, there was a greater increase in fat oxidation and a reduction in body fat compared to the controls. [115] Studies on obese individuals have also been able to show that replacing dietary fat sources with food rich in ω-3 LCPUFAs can cause an increase in the amount of weight loss in patients who are exercising or on weight loss diets. [116-118]

It is becoming evident that previously recommended low fat diets may not be ideal to curb the obesity trend. The intake of LCPUFAs and the omission of saturated and *trans*-

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fats should be recommended to obese individuals. [110] Despite this, very little attention has been paid to the anti-obesity effects of LCPUFAs.

2.3 Long chain polyunsaturated fatty acids and bone

Diet plays a crucial role in bone health. Calcium and phosphate are the most relevant minerals to bone as they make up 80-90% of the inorganic component of bone. [119] Proteins are essential for the formation of collagen in the organic matrix of bone. Several other minerals, vitamins and bioactive compounds in fruits and vegetables have also been shown to influence bone metabolism. [120] Although the mechanisms are not fully understood, several studies have reported beneficial effects of dietary LCPUFAs on bone.

2.3.1 In vitro studies

Many studies on the *in vitro* effects of LCPUFAs on bone cells have focused on metabolites of LCPUFAs, particularly prostaglandin E_2 (PGE_2) a metabolite of AA. It may be because it is commonly believed that the protective effects of LCPUFAs on bone are mediated through these metabolites. However, to fully understand the mechanisms involved in the interaction between LCPUFAs and bone, the action of the LCPUFAs themselves on bone requires investigation. A few relevant *in vitro* studies will be reviewed to examine our current understanding on the mechanisms involving LCPUFAs and bone cells.

2.3.1.1 Osteoblast studies

Studies on osteoblasts are conducted to ascertain whether or not LCPUFAs can modulate bone formation. A study performed by Coetzee *et al.* on MC3T3-E1 murine pre-osteoblasts found that AA decreased OPG secretion and increased RANKL secretion while DHA had no effect on either OPG or RANKL. [121] The authors propose that AA could reduce the OPG/RANKL ratio in osteoblasts and therefore could

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indirectly stimulate osteoclastogenesis. These results could suggest that AA may be detrimental to bone health. DHA showed no effect on the OPG or RANKL concentrations, although this is not to say DHA has no effect on the osteoblast but rather that the effect may be through a different mechanism. A later study by the same group showed that AA and DHA decrease alkaline phosphatase (ALP) activity in MC3T3-E1 cells after long term exposure of 14 days; however neither LCPUFA had an effect on mineralization. [122] DHA also decreased ALP activity after short term exposure which is of interest because of the role of ALP in mineralization. ALP is an important enzyme in hard tissue and is highly expressed during mineralization. [123] Therefore, it would be expected that a decrease in ALP activity would cause a decrease in mineralization. The duration of the experiments has been postulated as a reason for this unexpected finding. It was suggested that the cells may need to be exposed for longer periods in order to detect differences in mineralization. [122] However, more recently Choi *et al.* have shown that DHA can increase ALP activity in MC3T3-E1 cells. [124] The differences in these results can be explained by the different concentrations of fatty acids used in these two publications. Choi *et al.* made use of 0.1µM DHA while Coetzee *et al*. used 2.5-20µg/ml (≈ 7.5-60µM). The significantly higher concentration of DHA used by Coetzee *et al*. could be the cause of the decrease in ALP activity recorded. This could indicate that DHA has biphasic effects on osteoblasts.

Maurin *et al*. have shown that both DHA and AA can inhibit human primary osteoblast proliferation and apoptosis as well as cause these cells to withdraw from the cell-cycle.[125] This may be as a result of activating PPARs, which play a role in osteoblast formation. [21] The termination of proliferation and the cell-cycle are both preparative steps for osteoblast differentiation. [21] This could indicate that the LCPUFAs have a positive effect on osteoblast formation. In support of this, Watkins *et al*. have reported that LA, AA and EPA can increase the expression of Cbfa1 in rat foetal calvarial osteoblastic cells. [126] As stated earlier expression of Cbfa1 is the earliest identifiable event in osteoblastogenesis. Watkins *et al.* reported that AA had a greater effect than DHA. It was also noted that in another study on COX-2 knockout mice, the expression

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of Cbfa1 was lowered and that the addition of PGE_2 reversed this effect. [127] PGE_2 is formed from AA through COX and this could indicate that AA does in fact modulate osteoblast function through the formation of metabolites.

2.3.1.2 Osteoclast studies

It is commonly believed that the protective effects of LCPUFAs on bone are mediated through their inhibition of osteoclast function and formation, although the mechanisms are not fully understood. Zwart *et al*. were able to show that EPA could reduce RANKL-induced osteoclastogenesis in RAW 264.7 macrophages at 50µM. [128] In contrast, Yuan *et al*. showed that EPA (10µM), and AA (10µM), enhanced RANKL-induced osteoclastogenesis while DHA (10µM) showed an inhibitory effect. [129] The differences in experimental design can explain these contradictory results. Yuan *et al*. made use of bone marrow macrophages instead of the RAW 264.7 macrophages, as well as using 100ng/ml of RANKL as opposed to the 50ng/ml used by Zwart *et al*. Given that Yuan *et al.* have used twice as much RANKL and a lower concentration of EPA as Zwart *et al.* this could explain why EPA has differing effects on osteoclastogenesis in these two studies.

Rahman *et al*. were able to show some possible mechanisms through which LCPUFAs inhibit osteoclast function and formation, although this was only with the ω-3 LCPUFAs, DHA and EPA. [130] Both LCPUFAs inhibited osteoclastogenesis and resorption by osteoclasts derived from RAW 264.7 murine macrophages. Osteoclast specific genes such as TRAP, MMP-9, CTR and cathepsin K were down-regulated in the presence of DHA and EPA. The expression of c-Fos, an important osteoclast transcription factor, was also suppressed by exposure to the LCPUFAs. [130-132] NF-κB expression and the secretion of TNF-α were also shown to be reduced. TNF-α has been shown to have the potential to stimulate osteoclastogenesis. [133-135] Rahman *et al*. also noted significantly high levels of incorporation of the fatty acids tested into the cells. These results represent possible mechanisms through which LCPUFAs affect osteoclast functionality. In this study, DHA was shown to have more potent effects than EPA. [130] Cornish *et al.*

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showed that DHA had no effect on osteoclast formation and NF-κB expression; however this was at a much lower concentration than Rahman *et al.* [136] Cornish *et al*. reported an inhibitory effect of AA (2.5μg/ml (8μM)) on osteoclast formation but using much lower concentration of RANKL (10ng/ml) than Yuan *et al*.

Recently, Boeyens *et al*. have shown that AA (20μg/ml (65μM)) and DHA (20μg/ml (60μM)) could inhibit RANKL-induced osteoclastogenesis and resoption in RAW 264.7 murine macrophages. [137] This study found that the expression of the genes for cathepsin K and TRAP were down-regulated in the presence of the LCPUFAs tested. A down-regulation of these resorptive proteins could explain the decrease in resorption. Interestingly, DHA showed a stronger inhibitory effect than AA. These results on DHA are similar to those of Rahman *et al*. while the results on AA are similar to the findings of Cornish *et al.* [130, 136, 137] Although, Boeyens *et al.* used much higher concentrations of LCPUFAs than Cornish *et al*, it was reported that no cytotoxic effects were seen at these doses.

A recent study by Akiyama *et al*. made use of microarrays to identify the genes affected by DHA in murine bone marrow macrophages (BMMs). [138] The study found that of the genes that are usually up-regulated by RANKL, 4779 genes were down-regulated by the addition of DHA. These genes were involved in motility, adhesion, signalling and morphogenesis. Although all four of these factors are crucial for osteoclasts function, two are notable: adhesion which is required for bone resorption and morphogenesis which is required for the formation of osteoclasts (cell-to-cell fusion). PCR results confirmed that DC-STAMP, which is critical for cell fusion, was down-regulated by DHA. Several other osteoclast-specific genes were down-regulated by DHA. This could indicate that DHA may have a bone protective effect through the modulation of osteoclast-specific genes. [138]

While *in vitro* studies can be used to unravel the mechanisms involved in the interaction between LCPUFAs and bone, it is impossible to fully extrapolate these results into the biology of the human body. For this, *in vivo* studies are needed. A

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number of prominent *in vivo* studies will be discussed in the next section to attempt to link these results to what we understand from *in vitro* work. All these *in vitro* studies that have been discussed were performed on animal cell lines. To date, there have been no *in vitro* studies on the effects of LCPUFA on bone reported on human osteoclast cell lines. Table 2.1 contains a summary of selected *in vitro* studies.

Table 2.1: Summary of selected *in vitro* studies of LCPUFAs on bone cells

Publication	Cell lines used	Study design and tests conducted	Notable outcomes
2014, Boeyens *et al.* [137]	RAW 264.7 murine macrophages	**Fatty acids:** AA and DHA **Design:** RAW cells seeded at 7500cells/cm^2 with 15ng/ml RANKL. Exposed to 5-20µg/ml AA or DHA. **Tests:** TRAP staining, actin ring formation, resorption assays, western blot and PCR for resorption enzymes	-AA and DHA inhibited osteoclastogenesis, resorption and actin ring formation but DHA had a stronger effect -Gene and protein expression for two enzymes (cathepsin K and TRAP) were decreased with DHA showing a stronger effect
2011, Choi *et al.* [124]	RAW 264.7 murine macrophages MC3T3-E1 murine pre-osteoblasts	**Fatty acids:** EDHA and DHA **Design:** RAW cells seeded with 50ng/ml RANKL. MC3T3-E1 cells seeded with 50µg/ml ascorbic acid. Exposed to EDHA or DHA at 0.001, 0.01 or 0.1µM. **Tests:** MTT assay, Alkaline phosphatase activity, TRAP staining, western blot and RT PCR for markers of osteoclastogenesis	-EDHA inhibited osteoclastogenesis more strongly than DHA -EDHA reduced activity of JNK and NF-κB and suppressed expression of c-Fos and NFAT-c1 -DHA increased activity of alkaline phosphatase and BMP-2 in MC3T3-E1 cells
2010, Yuan *et al.* [129]	Murine bone marrow macro-phages (BMM)	**Fatty acids:** DGLA, AA, EPA and DHA **Design:** BMM flushed from femur of 6week old mice. Seeded into 48-well plate at 100000cells/cm^2 with 100ng/ml RANKL with or without PUFAs. Prostaglandins, COX and LOX inhibitor and DHA metabolites added to media. **Tests:** RT PCR for osteoclast markers and TRAP staining	-DHA inhibited osteoclastogenesis but EPA, DGLA and AA enhanced it -Enhancing effect was inhibited in the presence of COX inhibitors -Inhibitory effect of DHA reversed by LOX inhibitor -DHA inhibition may be due to decrease in DC-STAMP (responsible for cell-cell fusion) expression

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Table 2.1 continued…

2010, Zwart *et al.* [128]	RAW 264.7 murine macrophages	**Fatty acids:** EPA **Design:** Cells seeded in T-75 flasks with 50ng/ml RANKL with or without 50µM EPA. The cells were then split and plated according to the requirements of the test. **Tests:** Osteoclast formation, NF-κB activation by TNF-α induced osteoclastogenesis or exposure to modelled weightlessness	-EPA decreased RANKL induced osteoclast formation -NF-κB activation by TNF-α induced osteoclastogenesis or exposure to modelled weightlessness was reduced
2009, Coetzee *et al.* [122]	MC3T3-E1 murine pre-osteoblasts	**Fatty acids:** AA and DHA **Design:** Cells were seeded at 10^5 cells per well in 24-well plate for 48hrs to attach. Media was replaced with AA and DHA (2.5-20µg/ml) **Tests:** Alkaline phosphatase activity and mineralization	-AA inhibited alkaline phosphatase activity after long term exposure (14days) -DHA inhibited alkaline phosphatase activity after long term and short term (48hrs) exposure -Neither PUFA affected mineralization properties
2008, Cornish *et al.* [136]	Murine bone marrow macro-phages (BMM) RAW 264.7 murine macrophages Osteoblasts isolated from rat calvariae	**Fatty acids:** A number of SFA and ω-3 and ω-6 LCPUFAs **Design:** 10ng/ml RANKL and fatty acids were added at 0.1, 1 and 10µg/ml **Tests:** Analysis of gene expression by RT PCR, mevalonate enzyme pathway, osteoclastogenesis and bone resorption assay	-SFAs inhibited osteoclastogenesis in BMMs and RAW 264.7 cells -MUFAs and PUFAs were less effective than SFAs -FAs did not change expression of OPG or NF-κB or affect enzymes in mevalonate enzyme pathway -Receptors known to bind FAs were increased in osteoblastic and osteo-clastic cells
2008, Rahman *et al.* [130]	RAW 264.7 murine macrophages	**Fatty acids:** EPA, DHA and LA **Design:** Fatty acids were added at 6.25 to 50µg/ml with 50ng/ml RANKL **Tests:** Fatty acid incorporation, cell proliferation and apoptosis, TRAP activity and osteoclastogenesis, resorption, TNF-α secretion as well as western blotting for osteoclast markers and NF-κB assay	-Neither LCPUFA affected cell proliferation -DHA inhibited osteoclast formation, activity and functionality more than EPA -DHA also inhibited osteoclast specific genes (TRAP, cathepsin K, MMP-9, etc.) more than EPA -DHA inhibited TNF-α more than EPA -DHA significantly reduced NF-κB and p-38MAPK compared to EPA

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Table 2.1 continued…

2007, Coetzee *et al.* [121]	MC3T3-E1 murine pre-osteoblasts	**Fatty acids:** AA and DHA. PGE_2 and parathyroid hormone also used. **Design:** Cells were seeded at 50000 cells per well in 24-well plate for 24 hrs. AA or DHA (5-20µg/ml), or PTH or PGE_2 (10^{-8}M) was added. **Tests:** Determination of OPG and RANKL concentration	-AA inhibited OPG secretion by 25-30% -AA stimulated RANKL secretion -DHA had no significant effect on OPG and RANKL

2.3.2 In vivo studies

Studies on the *in vivo* effects of LCPUFAs focus mostly on the anti-inflammatory ω-3 LCPUFAs. This may be due to the fact that our Western diets usually contain high ratios of ω-6 to ω-3 LCPUFAs (greater than 15:1). [139] These high levels of ω-6 LCPUFAs may be responsible for promoting many diseases, such as cardiovascular disease, obesity, cancers, inflammatory and autoimmune diseases. [139] As most humans already receive adequate amounts of ω-6 LCPUFAs, researchers focus more on the effect of ω-3 LCPUFA high diets and supplementation to lower the ω-6:ω-3 ratio in our bodies and combat the adverse effects of our current diets.

2.3.2.1 Animal studies

Ovariectomized animals

Sun *et al.* reported on the effects of ω-3 LCPUFAs on bone health in female ovariectomized (OVX) mice. [11] The ovariectomy was done to mimic the conditions of menopause where oestrogen production from the ovaries ceases. After two months of being fed 5% corn oil (CO) or 5% fish oil and 0.5% corn oil (FO) diets, the mice were either sham-operated (mimicking the surgical procedure of the OVX without removing the ovaries) or OVX. The mice continued their diet as mentioned above for 16 weeks

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before bone density was measured. A week later they were sacrificed and serum lipid profile, RANKL expression and cytokine production by T-cells were measured. The study found that bone mineral density loss in OVX mice on the CO diet increased by 20% compared to the sham-operated mice on CO diet, while it only increased by 10% in OVX mice on the FO diet compared to sham mice on FO diets. The study also found a positive correlation between bone mineral density loss and RANKL production in OVX mice. Recently, Nakanishi *et al.* found that a fish oil diet suppressed the mRNA and protein expression of M-CSF, RANK, RANKL and other osteoclast transcription factors in OVX rats. [140] It can be suggested that dietary ω-3 LCPUFAs in fish oil could inhibit osteoclastogenesis, and therefore bone loss, in OVX rats through the inhibition of these transcription factors. NF-κB and TNF-α (both play a role in RANK signalling; See 2.3.3.5) activation was down-regulated by fish oil diets, suggesting these may be targets of LCPUFAs and that LCPUFAs can modulate RANK signalling. Rahman *et al.* have shown that conjugated LA could also inhibit the expression of bone resorption markers (NF-κB, RANKL, etc.) and increase the formation of osteoblastic markers in OVX mice. [141] OPG expression was also increased by conjugated LA. As LA is an ω-6 LCPUFA, this could indicate that both ω-3 and ω-6 LCPUFAs may have bone protective effects. However, the expression of pro-inflammatory cytokines was also increased by conjugated LA in this study. [141]

Chen *et al.* used the same model of the OVX mice to test the effects of endogenously produced ω-3 LCPUFAs on bone health. [142] The FAT-1 transgenic mouse model was used as it can endogenously convert ω-6 LCPUFAs to ω-3 LCPUFAs at a set rate. Wild-type (WT) mice were used as controls. This study found that endogenously produced ω-3 LCPUFAs lowered bone marrow adipogenesis thus allowing for an increase in osteoblast formation (osteoblasts and marrow adipocytes are both differentiated from bone marrow MSCs: See 2.1.1). “Switching” MSCs from adipogenesis to osteoblastogenesis would lead to increased bone formation and provides a novel drug target in the combat against bone disorders. Other bone parameters such as, bone

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mineral density, bone mineral content, etc. were also increased in FAT-1 mice compared to WT. [142]

Watson *et al*. examined the effect of the ratio of ω-6:ω-3 on bone health in OVX rats. [143] This study found that a lower ω-6:ω-3 ratio of 5:1 was more beneficial to bone than a ratio of 10:1. Bone mineral content and BMD readings showed that rats with higher DHA in their diet significantly lowered bone loss. This could indicate that the ratio of ω-6:ω-3 in the diet is crucial, and that a high ω-3 LCPUFA diet may be ideal. Sakaguchi *et al*. have shown that bone mass and strength were increased in OVX rats fed low-calcium diets with EPA than those on low-calcium diets without EPA. [144] This suggests that EPA could be involved in calcium absorption by the skeleton and may be a mechanism through which EPA and other ω-3 LCPUFAs exert their bone protective effects.

Weanling animals

A study by Al-Nouri *et al.* examined the effect of the ω-6:ω-3 ratio on certain bone health parameters in weanling rabbits. [145] In this study lower PGE_2 levels were observed with a lowered ω-6:ω-3 ratio. As PGE_2 is a metabolite of the ω-6 LCPUFA, AA, this could explain the observation. The study also showed that higher ω-3 LCPUFA levels in the bone marrows of rabbits on higher ω-3 LCPUFA diets. This is of interest because it demonstrates that ω-3 LCPUFAs can be incorporated into bone precursor cells formed in the bone marrow.

Lucia *et al* found a reduction in bone resorption markers with AA/DHA supplementation at an 8:1 ratio in weanling piglets. [146] The supplementation also resulted in increased femur mineral content and decreased bone PGE_2. Interestingly, the study found that PGE_2 (a metabolite of AA) supplementation had similar effects to LCPUFA supplementation. However, a combination of both LCPUFAs and PGE_2 showed a negative effect on bone. The addition of PGE_2 with LCPUFAs in the diet would lead to a higher concentration of PGE_2 in the body than if PGE_2 was added alone as the added

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LCPUFAs would be metabolized to PGE_2. The higher concentration of PGE_2 may be the cause of the negative effects seen in this study. This could further support the theory that the LCPUFAs do in fact act through their metabolites such as PGE_2. These results may also suggest that LCPUFAs have biphasic effects on bone health, as low levels of the metabolite PGE_2 were beneficial but seemingly high levels had negative effects.

Other studies have also shown that increasing ω-3 LCPUFAs and lowering the ω-6:ω-3 ratio in growing female rats, can have a positive effect on the bone mass and structure once these rats have reached maturity. [147] Increasing the ratio of ω-3 LCPUFAs may have a positive effect on bone by competing with ω-6 LCPUFAs for shared enzymes causing a reduction in ω-6 LCPUFA metabolites such as PGE_2 (See Figure 2.6). These results suggest that LCPUFAs can play a role in bone development and achieving peak bone mass.

2.3.2.2 Human studies

Older men and women

As stated earlier, decreases in oestrogen may attribute to the occurrence of osteoporosis in postmenopausal women. [45] Studies have been conducted on this high risk group to investigate whether LCPUFAs can help reduce the risk of fractures in post-menopausal osteoporosis. However, studies have yielded conflicting results. Tartibian *et al*. examined the effects of ω-3 LCPUFA supplementation and exercise on 79 healthy postmenopausal women. [148] This study showed that exercise in combination with LCPUFAs increased bone mineral density and serum bone turnover markers in these women after 24 weeks. However LCPUFA supplementation alone and exercise alone showed no significant effect on these factors. A prospective study by Virtanen *et al.* showed no association between hip fracture risk and increased LCPUFA diet. [149] As Virtanen *et al.* did not report whether the patients exercised regularly, this may explain their conclusion that LCPUFAs seemingly play no role in preventing hip fractures. It seems the effects of LCPUFAs are more prominent when used in combination with

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regular exercise. Interestingly though, Virtanen *et al.* did show that low intakes of total LCPUFAs, ω-6 LCPUFAs and LA may increase the risk of hip fractures in these patients.

Orchard *et al*. recorded similarly beneficial effects of ω-3 LCPUFAs to those of Tartibian *et al*. [150] This study found a positive association between lower risk of hip fracture and red blood cell ALA, EPA and ω-3 LCPUFA composition. A high ω-6:ω-3 ratio was also associated with an increased risk of hip fracture. In addition, this study showed that red blood cell LCPUFA composition is indicative of dietary LCPUFA intake. This was a case-controlled study and although steps were taken to choose appropriate controls, other explanations, such as exercise, cannot be ruled out as causal factors. However, a number of prospective studies have shown that, similar to Orchard *et al.*, LCPUFAs can help decrease the risk of hip fractures; ω-6 LCPUFAs (particularly AA) were also shown to be beneficial. [151-153] Interestingly, Farina *et al*. found that ALA could reduce the risk of hip fractures in both genders, while AA only reduced the risk of hip fractures in men. [153] This result may indicate that sex hormones play a role in the action of LCPUFAs.

Mangano *et al*. conducted a large cross-sectional study on 2135 elderly men and women (mean age 70years old). [154] The subjects were asked if they supplemented their diet with ω-3 LCPUFA, and BMD was assessed. The study found that there was a slight increase in femoral neck BMD associated with ω-3 LCPUFA supplementation. Total femoral BMD however, showed no change. Lumbar spine BMD was significantly increased in subjects taking ω-3 LCPUFA supplements. This is in line with previous findings of Kruger *et al*. which showed that elderly postmenopausal women (mean age 79.5years old) receiving calcium and LCPUFA supplements (600mg per day) had increased lumbar spine BMD. [155] However this study also showed increases in femoral BMD. These two studies were conducted over 36 months, however whereas Kruger *et al*. performed an intervention study, Mangano *et al*. performed an observational study and therefore there are many factors such as source and frequency of the LCPUFA supplementation, etc. that could lead to this conflicting result.

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Some studies have shown no effect of LCPUFAs on bone in postmenopausal women. Bassey *et al*. showed that there was no significant difference in total BMD and markers of bone turnover in postmenopausal women receiving Efacal (a pharmaceutical product containing GLA; 430mg), fish oil (70mg) and calcium (1g), compared to the control group. [156] This study was conducted over 12 months (as opposed to 36months in the previously mentioned studies) without a recommended exercise regimen and these factors may explain why no effects were seen. Furthermore, anti-osteoporotic treatments will generally affect areas rich in trabecular bone (such as the femoral neck and lumbar spine), as these sites have high rates of bone turnover. [21, 35] Therefore, measuring total BMD as a marker for anti-osteoporotic treatments may lack sensitivity to evaluate the effects of the treatment. In addition to this, GLA is an 18 carbon LCPUFA, while the LCPUFAs used in the studies which showed positive effects of LCPUFA on bone were 20 or 22 carbon LCPUFAs. This could indicate that the type of LCPUFA plays a role in its effects on bone health. It seems that the very long chain ω-3 PUFAs (20 or more carbons) could have beneficial effects on bone in postmenopausal subjects. [21]

Sharif *et al*. showed no changes in calcium, vitamin D and PTH levels in postmenopausal osteoporotic women receiving ω-3 LCPUFA supplementation (900mg per day) for 6 months. [157] Supplementation was shown to decrease bone resorption markers, however no effect was seen on bone formation markers. Shorter duration experiments seem to show lesser effects, indicating that the LCPUFAs may require long term exposure to affect bone health. The slow rate of bone formation (3-6 months) appears to affect the duration required for LCPUFAs to affect bone.

Hutchins-Wiese *et al*. in a pilot study on the effects of high doses of ω-3 LCPUFA supplementation (4g per day) in postmenopausal women have reported decreases in bone resorption markers after 3 months of intervention. [158] Although this was a short-term study and further testing is needed to assess whether these positive effects can be maintained, this study shows promise that positive effects can be seen with supplementation of high doses of ω-3 LCPUFAs.

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Growing youth

Beneficial effects of ω-3 LCPUFAs have been observed in growing youth as well. A study, known as the Northern Osteoporosis and Obesity Study (NO_2 Study) measured the ω-3 LCPUFA concentrations and bone health in 78 healthy males at baseline (16.7 years) and at age 22 and 24 to observe if any correlation existed between these two factors. [159] The study found a positive correlation between total and spinal BMD and ω-3 LCPUFA concentrations at age 22. There was also a correlation between changes in spinal BMD and ω-LCPUFA concentration between age 16 and 22. A negative correlation was seen between MUFAs and BMD, however AA and ω-3 LCPUFAs, particularly DHA, showed a positive association with BMD. These results show that AA and ω-3 LCPUFAs, DHA in particular, are associated with increases in BMD in young males.

A study by Harvey *et al*. was able to show that maternal LCPUFA status could affect bone development well into early childhood. [88] The study found that ω-3 LCPUFA concentrations in the mother were positively correlated with total BMD and lumbar spine BMD in the child at 4 years of age. These results further illustrate the importance of LCPUFAs in early childhood development and the importance of LCPUFAs in bone modelling.

The interpretation of *in vivo* results is difficult due to environmental, genetic and even dietary factors, such as the differences in the source of certain fatty acids, which are unique to specific regions. [160] Although the findings are generally promising, the exact effects of LCPUFAs on bone still remain unclear. Conflicting results that have been seen in the literature may be as a result of differences in the type, source, concentration and duration of use of the LCPUFA. Studies have shown that LCPUFAs may not just be beneficial for elderly subjects, but may be beneficial from the prenatal stage through to adolescence and into adulthood as well. This may indicate that LCPUFAs have positive effects on bone health throughout life and that a diet rich in LCPUFAs may be recommended for all.

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Table 2.2: Summary of selected *in vivo* animal studies of LCPUFAs on bone

Publication	Organism	Study design and tests conducted	Notable outcomes
2013, Chen *et al.* [142]	Wild-type mouse (WT) FAT 1 mouse (endogenously produce ω-3 LCPUFA from ω-6 LCPUFAs at set rate)	**Fatty acids:** Endogenous ω-3 LCPUFAs **Design:** At age 2 months, WT and FAT1 mice divided into 2 groups each, ovariectomized (OVX) and Sham with 10 mice in each group. The mice were fed high fat diets and sacrificed at 5months old **Tests:** Bone marrow adiposity and bone parameters (bone mineral density, bone mineral content, bone volume/total volume) in the distal femoral metaphysis were measured	-Endogenous ω-3 PUFAs lowered bone marrow adipogenesis, which provides a novel drug target -Bone parameters were also increased in FAT1 mice compared to WT
2012, Al-Nouri *et al.* [145]	Rabbit	**Fatty acid:** ω-3and ω-6 LCPUFA ratio **Design:** Weanling rabbits were assigned to five groups and fed on diets containing 70 g/kg of added oil with differing ω6/ω3 ratios (in square brackets) for 100 days as follow: soy bean oil (SBO control [8.7]), sesame oil (SO [21.8]), fish oil (FO [0.4]), DHA algae oil (DHA [0.6]), and DHA and AA algae oils (DHA/AA [0.7]) **Tests:** Bone marrow fatty acid profiles, PGE_2 levels and plasma alkaline phosphatase were analysed	-Rabbits on FO diet had highest ω-3 bone marrow LCPUFA profile and those on SBO had highest ω-6 LCPUFA profile -PGE_2 levels declined as ω-6/ω-3 ratio declined -Plasma alkaline phosphatase was higher in DHA/AA rabbits than in other groups
2011, Bonnet *et al.* [161]	Mouse	**Fatty acids:** ω-3 LCPUFA supplementation (EPA and DHA) **Design:** 30 female mice received a diet enriched in DHA or EPA or an isocaloric control diet from 3 to 17 months of age **Tests:** Bone turnover markers and bone histomorphometry were evaluated	-Trabecular bone volume in caudal vertebrae improved by 8 months but not thereafter -EPA improved femur cortical bone volume and thickness and reduced age-related decline of osteocalcin -EPA and DHA increased leptin however, only EPA further increased IGF-1 levels
2009, Shomali *et al.* [72]	Wistar rat	**Fatty acids:** EPA **Design:** 36 male rats, 2.5 months of age, were divided into six groups and treated with 0.9% NaCl (control), methylprednisolone (MPL) 7 mg/kg, once a week subcutaneously, MPL + alendronate 20µg/kg, twice a week subcutaneously and MPL + 80 or 160 or 320 mg/kg EPA, per day orally, for 6 weeks **Tests:** Serum and urinary parameters of bone metabolism were determined and bone histomorphometric analyses was performed	-None of the treatments had an effect on serum or urinary parameters measured -EPA, especially, at the dose of 160 mg/kg has beneficial effects on MPL-induced bone changes in rats

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Table 2.2 continued…

2003, Sun *et al.* [11]	Mouse	**Fatty acids:** ω-3 LCPUFAs **Design:** Sham and OVX mice were fed diets containing either 5% corn oil (CO) or 5% fish oil (FO) and 0.5% CO **Tests:** BMD was analysed. Serum lipid profile, RANKL expression and cytokine production by T-cells were also measured.	-BMD loss in OVX CO fed mice increased by 20% compared to Sham mice, while only increased by less than10% in OVX FO mice compared to Sham -BMD loss correlated with RANKL expression in OVX CO fed mice

Table 2.3: Summary of selected *in vivo* human studies of LCPUFAs on bone

Publication	Organism	Study design and tests conducted	Notable outcomes
2014, Hutchins *et al.* [158]	Human	**Fatty acid:** ω-3 LCPUFAs **Design:** 38 post-menopausal breast cancer survivors were randomly placed into two groups. One received 4g per of ω-3 LCPUFAs and the other received a placebo for 3 months. **Tests:** Bone turnover and inflammation markers were assessed	-No significant change was seen in inflammation markers after 3 months -ω-3 LCPUFA supplementation significantly reduced bone resorption markers
2013, Orchard *et al.* [150]	Human	**Fatty acid:** ω-3 and ω-6 LCPUFA **Design:** 201 incident hip fracture cases along with 199 additional incident hip fracture cases were randomly selected. Cases were matched on age, race, and hormone use with non–hip fracture controls. RBCs were analysed for LCPUFAs using gas chromatography **Tests:** Hip fracture risk was estimated for tertiles of RBC PUFA	-Positive association between lower hip fracture risk and RBC α-linolenic acid, EPA and ω-3 LCPUFA levels -Hip fracture risk almost doubled with the highest RBC ω-6/ω-3 ratio
2012, Virtanen *et al.* [149]	Human	**Fatty acids:** Dietary LCPUFAs **Design:** 75,878 women and 46,476 men free of osteoporosis were evaluated for dietary intakes by a food frequency questionnaire at baseline and several times during the follow-up **Tests:** Number of hip fracture cases were assessed to determine risk factor for hip fractures in relation to LCPUFA diet	-No association between increased LCPUFA diet and hip fracture risk was found -Low intake of total LCPUFAs, and particularly ω-6 LCPUFAs and linoleic acid, were found to increase the risk of hip fractures in women
2011, Farina *et al.* [153]	Human	**Fatty acids:** ω-3 and ω-6 LCPUFA **Design:** Associations between LCPUFA and fish intake, and hip fracture risk were analysed in 904 older adults (mean age 75yrs) for 17years **Tests:** Hip fracture risk was analysed	-A 54% reduction in hip fracture risk was seen in men and women with a high ALA intake -An 80% reduction in hip fracture risk was seen in men with a high AA intake

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Table 2.3 continued…

2011, Tartibian *et al.* [148]	Human	**Fatty acids:** ω-3 LCPUFA **Design:** 79 healthy post-menopausal women were separated into 4 groups (exercise+supplementation (E+S), supplementation (S), exercise (E) and control) for 24 weeks **Tests:** Lumbar spine and femoral neck bone mineral density, serum TNF-α, interleukin 6, PGE_2, oestrogen, osteocalcin, 1, 25-dihydroxyvitamin D3, C-telopeptide (a marker of resorption), PTH and calcitonin (CT) were measured at baseline, week 12 and 24	-Serum oestrogen, osteocalcin, 1, 25 Vit D, CT, lumbar and femoral neck BMD measures increased and the serum C-telopeptide, PTH, TNF-α, IL-6, and PGE2 decreased in E+S group after the 24 weeks -Lumbar and femoral neck BMD, oestrogen, osteocalcin, and CT were negatively correlated with TNF-α and PGE2 -PTH correlated positively and CT correlated negatively with IL-6
2007, Högström *et al.* [159]	Human	**Fatty acids:** ω-3 LCPUFA supplements **Design:** Cohort study of 78 young men with a mean age of 16.7 y at baseline **Tests:** Bone mineral density (BMD; in g/cm2) of total body, hip, and spine was measured at baseline and at 22 and 24 y of age. FA concentrations were measured in the phospholipid fraction in serum at 22 y of age	-Positive association between ω-3 LCPUFA concentrations and total and spine BMD at 22 y -Positive correlation between ω-3 LCPUFA concentration and changes in BMD of spine between 16 and 22y
1998, Kruger *et al.* [155]	Human	**Fatty acids:** GLA and EPA **Design:** 65 women assigned to GLA+EPA or coconut oil placebo diet, with 600mg calcium per day **Tests:** Markers of bone formation, degradation and BMD were assessed	-Femoral BMD increased 1.3% in treatment group and decreased 2.1% in placebo -Lumbar spine BMD increased 3.1% in treatment group -Subjects who switched from placebo to treatment saw a 2.3% and 4.7% increase in lumbar spine BMD and femoral BMD respectively

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2.3.3 Possible mechanisms of action of long chain polyunsaturated fatty acids on bone

Free fatty acids are not soluble in aqueous solutions and are therefore transported in the blood stream by serum albumin. [162] LCPUFAs can then be incorporated into the cell membrane which will cause an increase in membrane fluidity and may affect the biophysics of lipid rafts thereby modulating membrane proteins and downstream signalling pathways. [93] The incorporation of ω-3 LCPUFAs into the cellular membrane is known to affect toll-like receptor 4 (TLR4) which can cause anti-inflammatory effects by the down-regulation of NF-κB by preventing IKK degradation (Figure 2.7). [93] Down-regulation of NF-κB may have an effect on osteoclasts because of its known role in osteoclast formation and function through modulation of NFATc1 expression. Although LCPUFAs can affect intercellular mechanisms from incorporation in the membrane, they may be liberated from the cell membrane by cytosolic phospholipase A_2 ($cPLA_2$) and enter the cell. [93] Furthermore, LCPUFAs may be transported actively across the membrane through membrane proteins. [162] Inside the cell, LCPUFAs can act as natural ligands to key osteoclast regulators or be converted into mediators which exert various effects on osteoclasts.

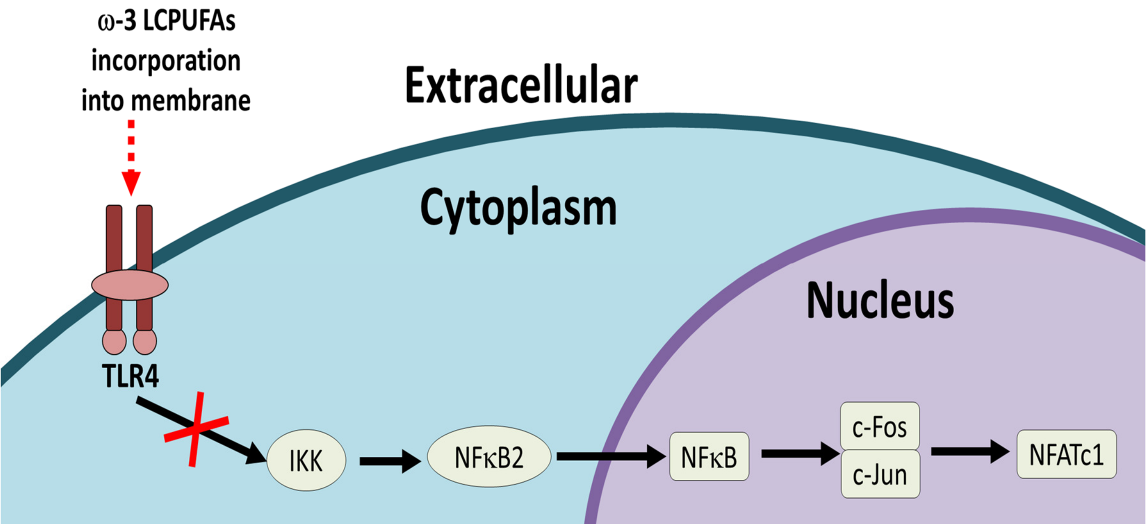

Figure 2.7: Effect of ω-3 LCPUFAs on TLR4. Incorporation of ω-3 LCPUFAs into the cell membrane can affect the biophysics of lipid rafts and therefore affect TLR4, a membrane protein. This may cause a down-regulation in NF-κB, which may affect NFATc1 expression and cause decreases in osteoclast formation and function. Image created with information from Mozaffarin *et al*. [93]

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2.3.3.1 Effect of long chain polyunsaturated fatty acids through PPAR activation

As was seen in some of the *in vitro* and *in vivo* studies reviewed, LCPUFAs can modulate osteoblast and osteoclast formation and function. PPARs are transcription factors expressed in osteoblasts and osteoclasts that are known natural ligands of LCPUFAs. [21] PPAR-γ is of particular importance because of its known role in both osteoclast and osteoblast formation (Figure 2.8). [163] As stated earlier, PPAR-γ can lead MSCs away from osteoblast differentiation and towards adipogenesis. This would undoubtedly lead to decreases in bone formation as fewer osteoblasts would be present. As osteoblasts produce RANKL and M-CSF which are required for osteoclast formation, bone remodelling as a whole can be affected by PPAR-γ. However, studies have revealed that PPAR-γ can control osteoclast differentiation by controlling c-Fos expression, a key regulator of RANK signalling (See 2.1.2). [163] PPAR-γ is known to promote osteoclastogenesis, through c-Fos, and consequently bone resorption. Wan *et al*. have demonstrated that PPAR-γ deficiency in the osteoclasts of mice selectively affected c-Fos activity in RANK signalling and that these animals developed osteopetrosis as a result. [164] This result reveals that PPAR-γ may play a crucial role in the control of osteoclast and osteoblast formation. ALA, LA and AA are known to be the most potent LCPUFA ligands of PPAR-γ, but other LCPUFAs such as DHA can act as ligands of PPAR-γ. [73] PPAR-α agonists are known to stimulate bone mass and OPG secretion. [165] It may be suggested that LCPUFAs could act through PPAR-α as well to affect bone mass, as some LCPUFAs such as DHA are known ligands of PPAR-α. [73] Ligands for both PPAR-α and PPAR-γ, such as AA and DHA, have been shown to counteract the bone destructive effects of PPAR-γ agonists. [73, 166] Therefore it can be suggested that LCPUFAs can modulate osteoclast and osteoblast formation through activation of PPAR-α and PPAR-γ.

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PPARγ
Mesenchymal stem cells
Haematopoietic stem cells
Adipocytes
RANKL
RANK
Osteoblast
cFos
Osteoclast precursor
c-Fms
M-CSF
Formation
Resorption

Figure 2.8: Effect of PPAR-γ on osteoclastogenesis and osteoblastogenesis. PPAR-γ stimulates haematopoietic stem cells (HSCs) to mature into osteoclast precursors through activation of c-Fos. Osteoclast precursors will form mature bone resorbing osteoclasts. PPAR-γ also affects osteoblastogenesis by promoting mesenchymal stem cells (MSCs) to differentiate into adipocytes instead of osteoblasts. PPAR-γ deficiency can lead to osteopetrosis (a condition of abnormally dense bone) indicating its vital role in controlling bone remodelling. Adapted from Wahli (2008). [163]

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2.3.3.2 Effect of long chain polyunsaturated fatty acids on calcium absorption

Calcium is a vital component of the inorganic matrix of bone. The absorption of calcium in the bone can therefore greatly affect the properties of bone. As previously stated, PTH plays a key role in calcium metabolism. It can stimulate the release of 1,25-dihydroxyvitamin D from the kidneys which acts on vitamin D receptors to increase intestinal calcium absorption and calcium resorption in bones. [167] Some studies have suggested that an increase in LCPUFAs in membrane phospholipids could be a mechanism through which 1,25-dihydroxyvitamin D increases calcium absorption in the gut. [21] The incorporation of LCPUFAs into the membrane phospholipids has effects on the structure, fluidity and membrane-bound proteins, such Ca^{2+} ATPase: an enzyme responsible for the active transport of calcium in the intestine. [21, 168] Mollard *et al*. reported that calcium content in the femur of piglets was increased with diets high in AA and DHA. [169] Some other *in vivo* studies mentioned previously also noted increases in bone mineral content with LCPUFA diets. [142, 144, 146] These studies further substantiate the claim that LCPUFAs can affect calcium uptake in bone.

2.3.3.3 Effect of long chain polyunsaturated fatty acids on bone through prostaglandins

Prostaglandins are paracrine and autocrine factors derived from LCPUFAs. PGE_2 is derived from the ω-6 LCPUFA AA, through the action of COX (See Figure 2.6), and is the major prostaglandin with known effects on bone remodelling. [73] PGE_2 can activate four different receptors that are of the G protein-linked receptor family, namely EP1, EP2, EP3 and EP4, and are all expressed on the surface of osteoclast precursors. [170-172] Kobayashi *et al*. have shown that the effect of PGE_2 on RANKL-induced osteoclastogenesis is mediated through EP2 and EP4 receptors. [172] This study has also shown that EP2 and EP4 receptors have the same intracellular effects as calcitonin (a known inhibitor of bone resorption) receptors, namely increasing adenosine 3',5'-cyclic mono-phosphate (cAMP) and protein kinase A (PKA) activity. [172] However, *in vitro* studies have shown that expression of the EP2 and EP4 receptors could be down-

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regulated during RANKL-induced osteoclastogenesis. [172] EP3 receptors have been shown to inhibit cAMP, possibly promoting osteoclastogenesis as its effect is opposite to that of calcitonin receptors, but its regulation is not well described. [172] Ono *et al.* reported PGE_2 to have biphasic effects on osteoclast formation, with short term exposure (5-7 days) causing osteoclast inhibition and long term exposure (7-8 days) increasing osteoclast activity. [171] This may result as EP2 and EP4 receptors are still active in osteoclast precursors, causing inhibition, but are down-regulated over time leading to the promotion of osteoclastogenesis. [171, 172] Prostaglandin E_3 (PGE_3) is derived from the ω-3 LCPUFA EPA and has similar functions to PGE_2 in bone. [73] Hawcroft *et al.* have shown that PGE_3 can bind to the PGE_2 receptor, EP4, in human colorectal cancer cells, but with reduced efficacy and affinity than PGE_2. [173] PGE_3 may bind to the same receptor in osteoclasts resulting in similar effects to PGE_2 but with reduced efficacy.

It has been reported that high levels of PGE_2 in bone may have negative effects on bone health while low level exposure may have beneficial effects. [174] Lucia *et al.* reported that dietary LCPUFA can increase bone mineral content while decreasing bone PGE_2 in weanling piglets. [146] Al-Nouri *et al.* observed that lower PGE_2 levels were associated with lower ω-6:ω-3 LCPUFA ratios. [145] These results could indicate that LCPUFAs can affect bone remodelling by affecting the amount of PGE_2 present in the bone. This may be due to the fact that AA and EPA are catalysed to PGE_2 and PGE_3 respectively, by the same enzyme; COX (See Figure 2.6). There are two known isoforms of the COX enzyme, COX-1 and COX-2. [175] Both isoforms metabolize AA, but only COX-2 has been shown to metabolize EPA at 30% the efficiency of AA. [176] In addition, EPA can inhibit the oxygenation of AA by competing for binding of the COX enzymes and therefore lower the amount of PGE_2 in the tissue. [176] Increasing the amount of ω-3 LCPUFAs in the diet could therefore have the effect of lowering the amount PGE_2 which could possibly explain the bone protective effects of ω-3 LCPUFAs.

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2.3.3.4 Effect of long chain polyunsaturated fatty acids through IGF-I modulation

Li *et al*. have shown that dietary ω-3 LCPUFAs can increase serum levels of insulin growth factor binding protein (IGFBP) -3 in growing male rats. [177] IGFBP-3 is a key modulator of insulin-like growth factor (IGF)-I which is an important regulator of bone formation and growth. [73] More than 90% of IGF-I in the serum is bound to IGFBP-3. [178] IGF-I stimulates the proliferation of osteoblast precursors increasing the potential for the formation of mature osteoblasts. [73] IGF-I has also been shown to be necessary for the normal interaction between osteoblasts and osteoclasts as it may display autocrine and paracrine effects during remodelling. [179] In addition, Damgaard *et al*. have shown a positive association between DHA status and serum IGF-I levels in adolescent boys. [180] By increasing the levels of IGF-I, LCPUFAs may increase osteoblastogenesis and bone formation, and this may be a method through which they exert their bone protective effects.

2.3.3.5 Effect of long chain polyunsaturated fatty acids through TNF-α modulation

TNF-α is a cytokine involved in inflammatory tissue destruction and is known to play a role in osteoclastogenesis. [13] Many different cell types, such as macrophages and osteoblasts, are known to secrete TNF-α. [13] When TNF-α binds to the TNF-receptor (TNFR) on osteoclast precursors it can activate intracellular signalling pathways, such as the JNK pathway, and the NF-κB pathway through the phosphorylation and deactivation of IKKs (similar to RANK-RANKL interaction) (Figure 2.9). [13, 181] These pathways both activate c-Fos which interacts with the NFATc1 promoter and triggers the completion of the differentiation of osteoclasts. [181] These same pathways are stimulated during RANKL-induced osteoclastogenesis (See 2.1.2). TNF-α has been shown to stimulate its own secretion and that of IL-1, another stimulator of osteoclastogenesis. [181]

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Figure 2.9: TNF-α binding to TNFR in osteoclasts. TNF-α can activate the NF-κB pathway similar to RANKL. TNF-α can also activate c-Fos through the JNK1 pathway. It is also known to up-regulate its own expression and stimulate IL-1 which in turn stimulates osteoclast formation through ERK1. Adapted from Boyce *et al.* [181]

However, IL-1 does not stimulate c-Fos, but rather activates osteoclastogenesis directly through ERK1 in osteoclast precursors in which c-Fos has already been activated. [181] Skuladottir *et al.* have shown that ω-3 LCPUFAs can decrease the expression of TNF-α in RAW 264.7 murine macrophages. [182] Additionally, Bhattacharya *et al.* demonstrated

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that high fish oil diets reduced TNF-α levels in female rats.[183] The modulation of this cytokine represents another target for LCPUFAs, especially ω-3 LCPUFAs.

2.3.3.6 Effect of long chain polyunsaturated fatty acids through lipid mediators

Resolvins, lipoxins, leukotrienes, protectins, etc. are anti-inflammatory lipid mediators formed by LOX metabolism of LCPUFAs (See Figure 2.6). Lipoxins and resolvins are known to inhibit inflammatory bone resorption and thereby present another mechanism through which LCPUFAs could protect against bone loss.[73] Hasturk *et al*. have reported that resolvin E1 reduced inflammation and osteoclast-mediated bone resorption in patients with perionditis.[184] Furthermore, Herrara *et al*. have shown that resolvin E1 could reduce osteoclastogenesis and resorption pit formation in BMMs.[185] Resolvins are known to down-regulate the expression of pro-inflammatory cytokines such as PGE_2.[186] As high levels of PGE_2 are known to stimulate osteoclast formation and resorption, a decrease in PGE_2 may explain the bone protective effects of resolvin E1. Lipoxins have been shown to down-regulate TNF-α induced NF-κB activation, and this could represent a method through which they can inhibit osteoclast formation and resorption.[187]

2.3.3.6 Effect of long chain polyunsaturated fatty acids through nitric oxide

Nitric oxide (NO) is a free-radical that is synthesized from oxygen and L-arginine by NO synthase (NOS).[73] There are three known isoforms of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS).[188-190] nNOS and eNOS are constitutively expressed, and while nNOS knockout mice have decreased osteoclast formation, eNOS and iNOS knockout mice show normal osteoclast formation.[191-194] Constitutive low-levels of NO are believed to be involved in osteoblast differentiation while high-levels of NO may act as a mediator for cytokine-induced resorption in osteoclasts and play a role in pathological conditions such as osteoporosis.[73, 195] High levels of NO may also decrease TGF-β and therefore inhibit osteoblast formation.[196] AA has been shown to up-regulate iNOS in human osteoblastic cells and may therefore

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decrease osteoblast formation by increasing NO production. [197] DHA has been shown to down-regulate iNOS and may therefore inhibit osteoclast activity and stimulate osteoblast formation by decreasing NO production. [198]

2.4 Conclusion

Osteoporosis is a prevalent and debilitating bone disorder. Current drug therapy can prove costly and side effects are common. Preventing or slowing down the occurrence of osteoporosis, through proper diet and lifestyle would be an ideal method of combating this disease. LCPUFAs have been shown to have many beneficial effects in the body such as assisting in neonatal development, improving heart health as well as possessing anti-cancer and anti-obesity effects. Beneficial effects of LCPUFAs, particularly fish oils rich in ω-3 LCPUFAs, on bone health have been reported for many years, although the mechanism of action LCPUFAs favour still remains unclear. Combinations of two or more of the listed mechanisms could be involved in the bone protective effect of LCPUFAs on bone. It is evident, though, that LCPUFAs can modulate the function and formation of osteoblasts and osteoclasts. Although *in vitro* and animal studies have shown that LCPUFAs may have bone protective effects, human studies have been promising but less conclusive on the matter. Environmental factors, as well as the wide range of sources of LCPUFAs may contribute to the confounding results seen in human studies. Adding to this, the *in vitro* effects of LCPUFAs have not been reported in a human osteoclast cell model. Studying the effects of LCPUFAs on human osteoclasts could give a better understanding of the interaction between LCPUFAs and osteoclasts and form the backbone for future research.

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CHAPTER 3

MATERIALS and METHODS

Preliminary experiments were conducted at the Mellanby Bone Centre, University of Sheffield. All subsequent experiments were conducted in the cell culture laboratories of the Department of Physiology, Faculty of Health Sciences, University of Pretoria. The hippo tusk was sectioned at the Department of Oral Pathology and Oral Biology (University of Pretoria). Light microscopy was done at the Laboratory for Microscopy and Microanalysis, University of Pretoria.

3.1 General laboratory procedures

3.1.1 Reagents and materials

Antibiotic-solution containing penicillin (100U/ml), streptomycin (100µg/ml) and fungizone (0.25µg/ml) was supplied by Highveld Biological (Johannesburg, South Africa). Histopaque® (density - 1,077g/ml), DHA, AA and all other chemicals of analytical grade were obtained from Sigma-Aldrich Inc. (St Louis, MO, USA). Alpha-minimum essential medium (α-MEM) without nucleic acids was purchased from GIBCO (Invitrogen Corp, Australia). Amersham (Little Chalfont, UK) supplied foetal bovine serum (FBS). RANKL was acquired from Insight Biotechnology (Middlesex, UK) and R&D Systems (Minneapolis, MN, USA) supplied M-CSF. Cell cluster plates were obtained from Lasec (Cape Town, South Africa). Glass coverslips were purchased from Lasec (Cape Town, South Africa) or Labotec (Midrand, South Africa) depending on availability. All components for the magnetic separation were supplied by MiltenyiBiotec (Bergisch Gladbach, Germany). Primary antibodies for western blotting were purchased from Abcam (Cambridge, UK) while secondary antibodies were purchased from Life Technologies (Carlsbad, CA, USA). Reverse transcriptase was

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purchased from New England Biolabs (Ipswich, MA, USA). Oligo (dT) primers were purchased through InqabaBiotec (Pretoria, South Africa). Other PCR reagents (dNTPs, DNA polymerase, DNA ladder, loading dye, etc.) were supplied by KAPA Biosystems (Cape Town, South Africa).

3.1.2 Preparation of stock solutions

Table 3.1: Preparation and storage conditions of stock solutions used throughout this study

Compound	Preparation and storage of stock solution
LCPUFAs: Arachidonic acid (AA)* Docosahexaenoic acid (DHA)*	100mM in 100% ethanol Aliquots stored at -80°C in the dark
RANKL	30µg/ml in culture medium Aliquots stored at -20°C in the dark
M-CSF	25µg/ml in culture medium Aliquots stored at -80°C in the dark
Antibodies: GAPDH TRAP MMP-9	1mg/ml in PBS Aliquots stored at -80°C in the dark
Antibody: Cathepsin K	0.5mg/ml in PBS Aliquots stored at-80°C in the dark
Primers: GAPDH TRAP CTSK MMP-9 NFATc1 c-Fos CA2 DC-STAMP RANK	100µM in nuclease free water From 100µM stock: 10µM of forward and reverse primer prepared in nuclease free water Aliquots stored at -20°C in the dark

* When diluted, ethanol concentrations in cell culture medium did not exceed 0.08% (v/v)
Ethanol at 0.08% did not show any harmful effects on the cells tested in our laboratory.

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3.1.3 Preparation of dentine discs

A hippo tusk was kindly donated by Dr Gerhard Steenkamp (Veterinary Hospital, Onderstepoort). The tusk was sectioned into thin slices (180-200µm) using the Struers Axitom-5 cut-off machine (Cleveland, OH, USA). Sectioning was done at the Department of Oral Pathology and Oral Biology, University of Pretoria. The slices were soaked in distilled water for at least an hour and then punched into 6mm discs using a standard paper punch. The discs were sonicated twice for 10mins and then submerged in 70% ethanol and stored at 4°C until needed. Before culturing cells on the dentine discs, they were rinsed twice in PBS and then placed in culture medium and kept at 37°C overnight.

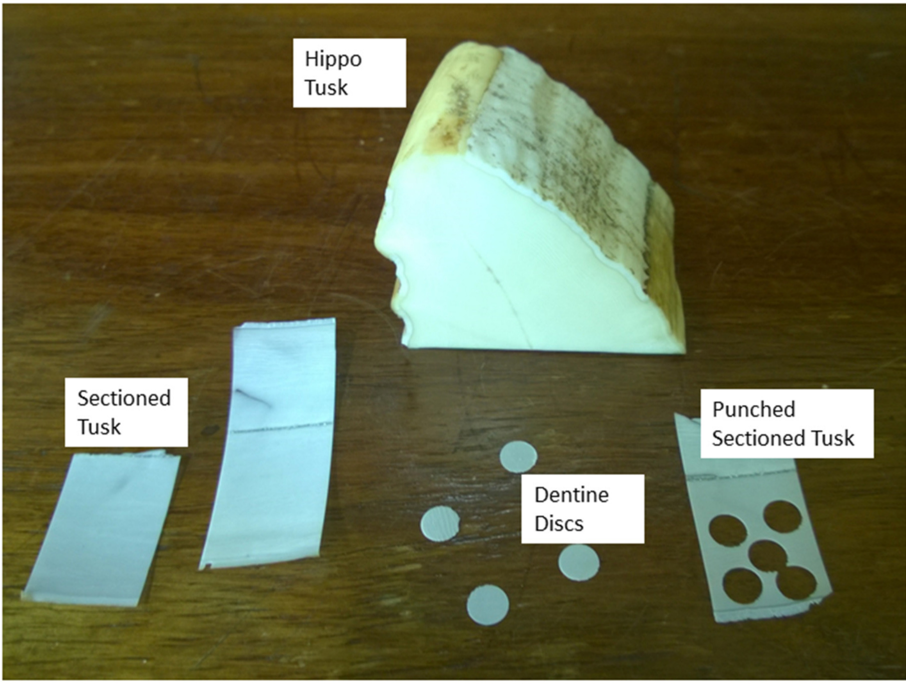

Figure 3.1: Preparation of dentine discs. In the image, different stages of the preparation of the dentine disc are seen: a piece of hippo tusk, slices of sectioned tusk, a punched sectioned tusk, and the final dentine discs that the osteoclasts are cultured on.

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3.1.4 Preparation of buffers and solutions

Table 3.2: Preparation and storage conditions of buffers and solutions used throughout this study.

Buffers and solutions	**Preparation and storage of buffers and solutions**
Phosphate buffered saline (PBS)	80g NaCl, 2g KCl, 2g KH_2PO_4 and 10,5g Na_2HPO_4 in 1L distilled water for 10xPBS stock. pH adjusted to 7.4. Stock diluted 10 times in distilled water and autoclaved. Kept at 4°C until needed.
Buffer1 (For preparation of CD14+ monocytes)	PBS supplemented with 2mM EDTA. Prepared fresh when needed.
Buffer2 (For preparation of CD14+ monocytes)	PBS supplemented with 2mM EDTA and 0.5% BSA. Prepared fresh when needed.
pNPP solution	Substrate buffers (0.0088g/ml L-ascorbic acid, 0.046g/ml $C_4H_4Na_2O_6$, 0.025g/ml and 4-nitrophenylphosphate disodium) hexahydrate and reaction buffer (1.0M acetate, 0.5% Triton X-100, 1M NaCl, 10mM EDTA adjusted to pH 5.5) were prepared. 12.5% L-ascorbic acid, 12.5% l $C_4H_4Na_2O_6$, 12.5% 4-nitrophenylphosphate disodium hexahydrate, 25% reaction buffer and 37.5% distilled water to prepare working solution. Substrate buffers and reaction buffers prepared prior to use and stored at 4°C until needed. Working solution prepared fresh when needed.
Acetate-tartrate buffer	5.44g $CH_3COONa_2 \cdot 3H_2O$ and 4.6g $C_4H_4Na_2O_6$ in 200ml distilled water. pH adjusted to 5.2 with 50ml of 1.2% $C_2H_4O_2$. Prepared fresh when needed.
Naphthol-dimethlyformamide solution	0.02g napthol AS-Bl phosphate and 1ml $HCON(CH_3)_2$ in 50ml acetate-tartrate buffer. Prepared fresh when needed
Pararosaniline solution	2ml pararosaniline and 2ml of 4% $NaNO_2$ in 50ml acetate-tartrate buffer. Prepared fresh when needed.

3.2 Cell culture

Highly purified CD14+ monocytes isolated from peripheral blood and grown in the presence of differentiation factors (RANKL and M-CSF) have the ability to differentiate into giant bone resorbing cells that display the phenotypic and genotypic

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characteristics of osteoclasts. [2, 199, 200] CD14+ monocytes were therefore used to generate human osteoclasts in this study.

Ethics approval for the proposed study on human CD14+ monocytes was obtained in 2012 from the Faculty of Health Sciences Research Ethics Committee of the University of Pretoria (ethics approval number: S154/2012) (See Appendix A).

3.2.1 Preparation of CD14+ monocytes

Blood was collected from healthy male donors (age 18-32), after informed consent was given (See Appendix B), and diluted 1:1 in buffer1 (See Table 3.2). The blood was then layered carefully on a Histopaque® gradient and centrifuged at 450xg for 30 minutes at 20°C without brake in a Hettich Rotixa 120R centrifuge (Kirchlengern, Germany). The peripheral blood mononuclear cells (PBMCs) were then carefully collected with a 1ml pipette from the layer between the plasma and the Histopaque® layers. The cells were washed once with PBS containing 2mM EDTA and centrifuged again at 300xg for 10mins at 20°C and then washed again and centrifuged at 200xg for 10mins. The cells were then counted, resuspended in buffer1 and centrifuged again for 10mins at 300xg. CD14+ cells were then sorted by magnetic separation as follows:

The cells were resuspended in 80µl of buffer2 (See Table 3.2) per 10^7 cells. Thereafter 20µl of MACS® MicroBeads per 10^7 cells was added. The suspension was mixed carefully and incubated for 15 minutes on ice, after which the cells were washed by adding 1-2ml of buffer2 per 10^7 cells and centrifuged at 300xg for 10 minutes. The cells were then resuspended in 500µl of the buffer2 and passed through a magnetic separation column. The column was washed three times with 500µl of buffer2. The cells which pass through were the unlabelled cells and the labelled CD14+ monocytes remained in the column. One ml of the buffer2 was added and the magnetically labelled cells were flushed out of the column. The CD14+ monocytes were then counted and seeded into cell cluster plates as required.

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3.2.2 Cell culture maintenance

Cells were propagated in complete growth medium which contains α-MEM as base with 10% FBS added. All media was supplemented with 100U/ml penicillin, 100µg/ml streptomycin and 0.25µg/ml fungizone. Cells were propagated and maintained in a humidified atmosphere of 37°C and 7% CO_2 as previously described by Agrawal *et al*. (this concentration of CO_2 is used as the human osteoclasts have been found to prefer an acidic environment). [2] The CD14+ monocytes were either seeded on glass or dentine discs (See 3.1.3) as it has been found that they do not grow ideally on plastic surfaces. [2] Glass coverslips were sterilized by baking at 180°C for 2hrs and then allowed to cool at room temperature until needed.

3.3 General methods for experiments

Following trypan blue exclusion, CD14+ monocytes were seeded at 130 000 cells/cm^2 in 96- or 24-well plates, and exposed to differentiation factors, RANKL (30ng/ml) and M-CSF (25ng/ml) and allowed to attach for 48hrs. Previous work done in our laboratories on the effect of LCPUFAs in RAW 264.7 murine macrophages showed that concentrations of LCPUFAs in the range of 20-80µM had an inhibitory effect on osteoclast formation and resorption. [137] As no other work has been reported of the effects of LCPUFAs on human CD14+ monocytes, this concentration range was chosen for this study. Therefore, cells were exposed to either LCPUFA (20, 40, 60 and 80µM) or the vehicle control (<0.08% ethanol) in two ways: from day 3 if the experiment was on differentiating osteoclasts or when resorption was first seen (day 11-14) if the experiment is on mature osteoclasts. [2] The concentration of ethanol in the vehicle control showed no harmful effects on the cells. Medium, differentiation factors and LCPUFAs were replaced every 2-3 days. In order to determine when the control cells reached the resorptive stage, cells were grown on extra dentine discs and removed daily from day 11 onwards. The dentine discs were stained with toluidine blue and checked for the presence of resorption pits under a light microscope (Figure 3.2). The cells were allowed to differentiate for an extra 7 days after resorption is first seen

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(± 21 days). Three independent experiments were conducted with cells harvested from different donors for each test. The results of the LCPUFA exposed groups were compared to those of the vehicle control.

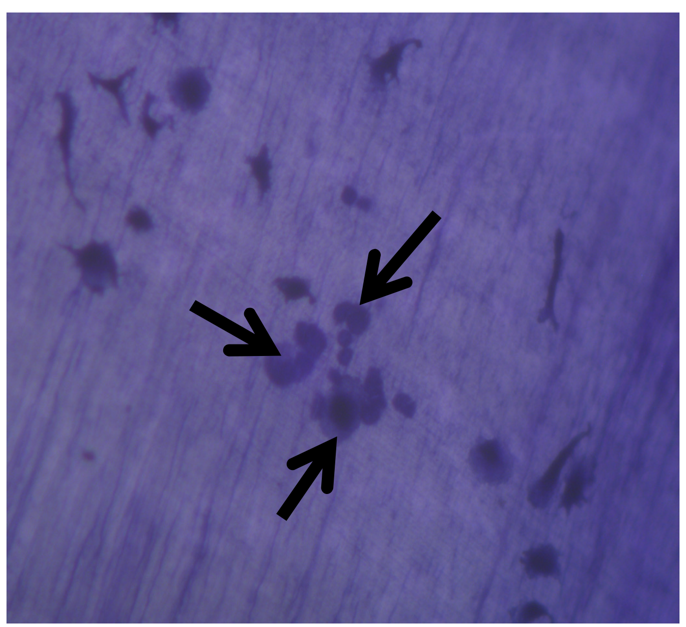

Figure 3.2: Toluidine blue stained dentine disc. Resorption pits are indicated with arrows.

3.3.1 Osteoclast formation

Following trypan blue exclusion, cells were seeded at 130 000 cells/cm^2 in triplicate on glass coverslips in sterile 96-well plates with differentiation factors, RANKL (30ng/ml) and M-CSF (25ng/ml). Cells were exposed to LCPUFAs at 20-80µM for experiments on both differentiating osteoclasts and mature osteoclasts. Culture media, differentiation factors and LCPUFAs were replaced every 2-3 days. The experiments were terminated 7 days after the onset of resorption.

3.3.1.1 Quantification of tartrate-resistant acid phosphatase (TRAP) in conditioned media

An early marker of osteoclastogenesis is the export of the lytic enzyme TRAP, which is necessary for the resorption of the bone matrix. [200] It can therefore be used as an indicator for the presence of mature osteoclasts. [200] During medium changes and at

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the end of culture, 20µl of medium was removed from each well and TRAP activity in the conditioned media was measured by adding 80µl pNPP solution (See Table 3.2). [200] The mixture was incubated for 60mins after which the reaction was stopped with 100µl of 0.3M NaOH and immediately quantified by measuring optical absorbance at 405nm with 650nm as the reference using an Epoch Micro-plate Spectrophotometer (BioTek, Winooski, VT, USA). TRAP converts the pNPP solution into a coloured reaction product, the intensity of which can be an indicator of the amount of TRAP present, which in turn could indicate the amount of osteoclasts present.

3.3.1.2 Quantification of mature tartrate-resistant acid phosphatase positive (TRAP+) stained osteoclasts

At the end of the culture period, osteoclasts were fixed with 3.7% (v/v) formadehyde in PBS for 5mins and stained for the presence of TRAP using a modified TRAP staining protocol as previously described. [2] In short: the fixative was removed and a warm 100µl acetate-tartrate buffer (See Table 3.2) was added to the cells for 5min at 37°C. The buffer was removed and 100µl of a naphthol-dimethylformamide solution (See Table 3.2) was added and the cells were incubated at 37°C for 30mins. After incubation, the naphthol-dimethylformamide solution was removed and 100µl of pararosaniline solution (See Table 3.2) was added. After 15mins incubation at 37°C, the cells were counter-stained with haematoxylin for 40 seconds, rinsed, air-dried and visualised by light microscopy. Osteoclasts appear as large multinucleated cells staining red. [7] The effect of the LCPUFAs on osteoclast formation was determined by counting the number of TRAP-positive stained cells with 3 or more nuclei in each well. [130] Photomicrographs were taken with a Zeiss Axiocam MRc5 camera attached to a Zeiss Axiovert40 CFL microscope (Zeiss, Göttingen, Germany).

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3.3.2 Quantification of resorption pit formation on dentine discs

Following trypan blue exclusion, cells were seeded at 130 000 cells/cm^2 in triplicate on dentine discs in sterile 96-well plates with differentiation factors, RANKL (30ng/ml) and M-CSF (25ng/ml). This experiment was conducted on mature osteoclasts exposed to LCPUFAs at 20-80µM. Culture media, differentiation factors and LCPUFAs were replaced every 2-3 days. The experiments were terminated 7 days after the onset of resorption.

The ability to resorb bone is a primary function of osteoclasts. [7] At the end of culture period, the osteoclasts were fixed with 3.7% (v/v) formadehyde in PBS for 5mins and stained as mentioned in 3.3.1.2. Haematoxylin stained the resorption pits on the dentine discs. Dentine discs were imaged using a Zeiss Axiocam MRc5 camera attached to a Zeiss SteREO Discovery.V8 microscope (Zeiss, Göttingen, Germany). A method known as point counting was used to determine the percentage resorption. A grid was created on the image using ImageJ software, [201] and resorption pits crossing the intersecting points on the grid were counted (See 3.3.2.1). This is an indirect method for determining percentage resorption. [2]

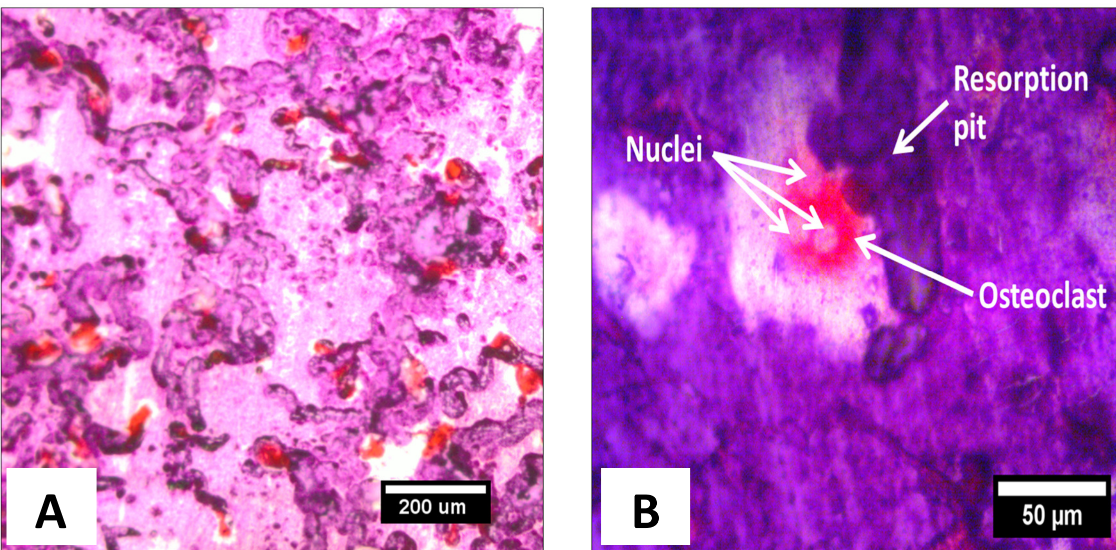

Figure 3.3: Osteoclasts on dentine discs. **A.** Enlarged image of dentine disc showing a clear view of osteoclasts (red dots) and resorption trails (dark purple areas). **B.** A single bone resorbing osteoclast resorbing dentine. Three nuclei are clearly visible, showing that counting TRAP+ cells with 3 or more nuclei as mature osteoclasts is valid.

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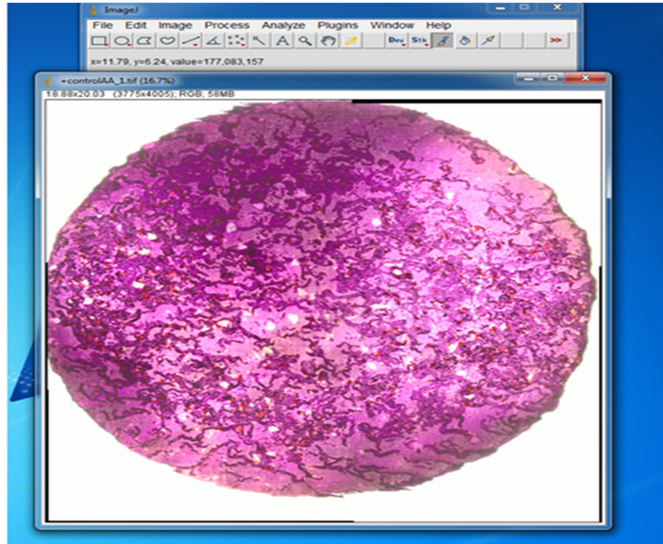
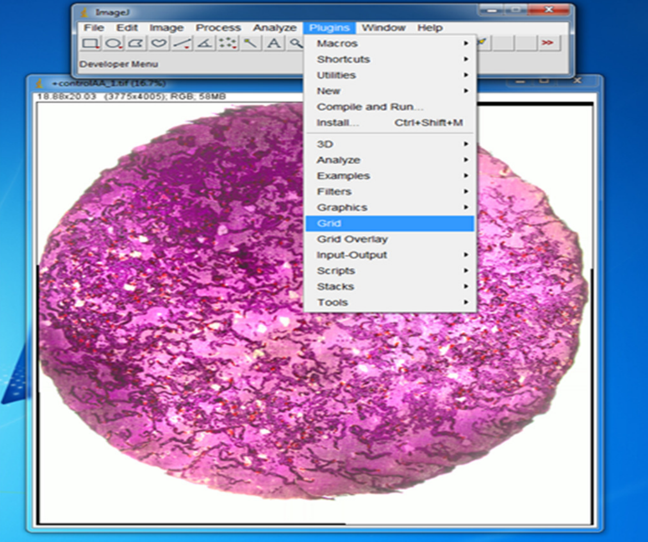
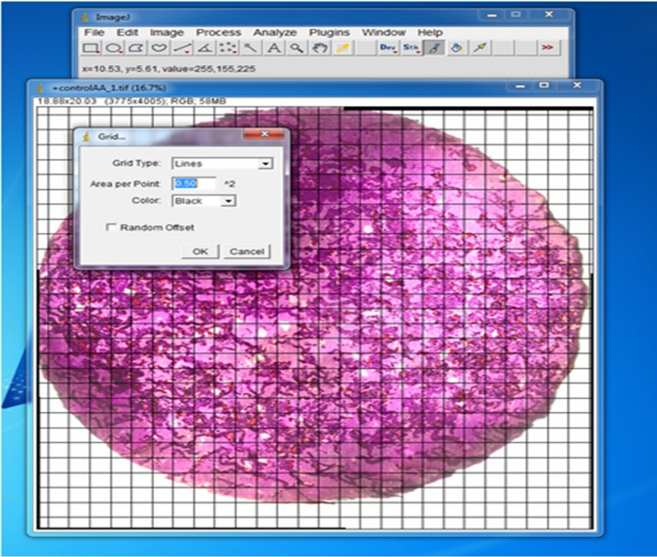
3.3.2.1 Point counting



1. Open dentine disc image in ImageJ. ImageJ is freeware that can be downloaded from: www.imagej.nih.gov/ij



2. Download and open grid plugin. www.rsd.info.nih.gov/ij/plugins/grid.html



3. Choose the size of the grid. The number is arbitrary as long as it is consistent with all the images being analysed. Insure there are at least 20 horizontal and vertical lines across the disc.

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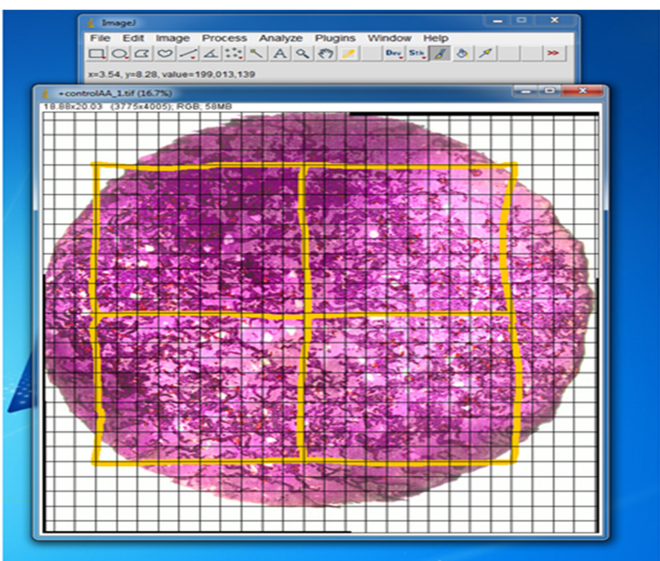
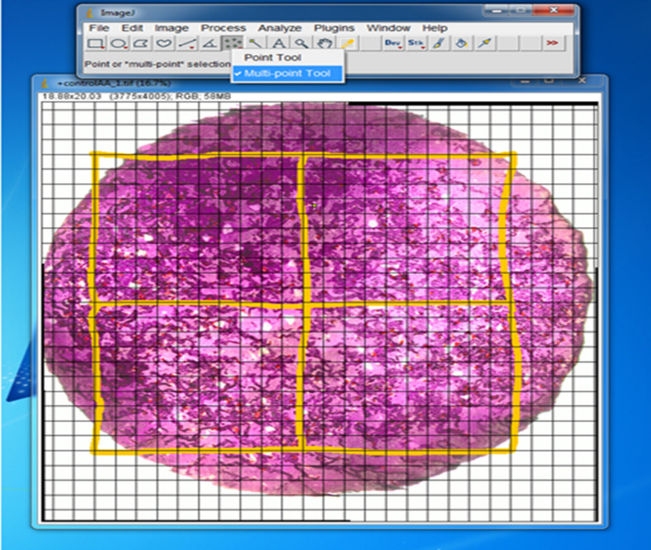
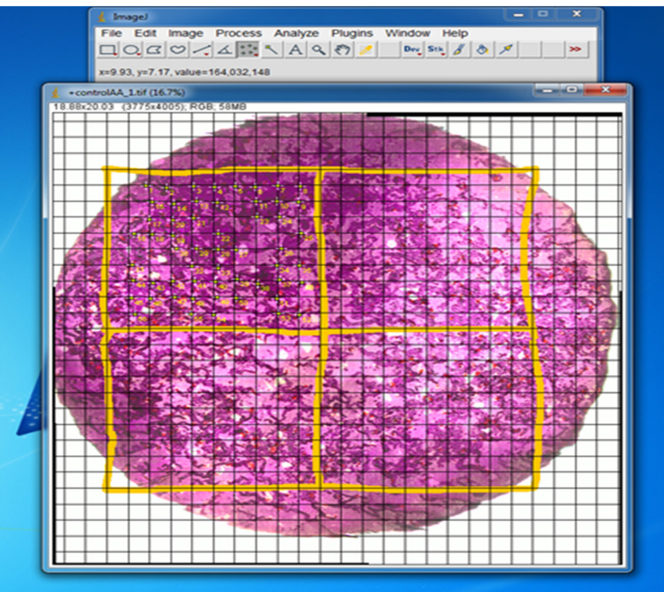


4. Use the drawing function on ImageJ to mark four separate 10x10 grids on the dentine disc. Ensure the four grids are identical on all discs that are analysed



5. Right click on the pointing tool and select the multipoint tool. Multi-point is used to assist with counting.



6. Click on a resorption pit if it touches an intersecting line within the 10x10 grid, i.e. an intersecting horizontal and vertical line. These are known as the points on the grid.

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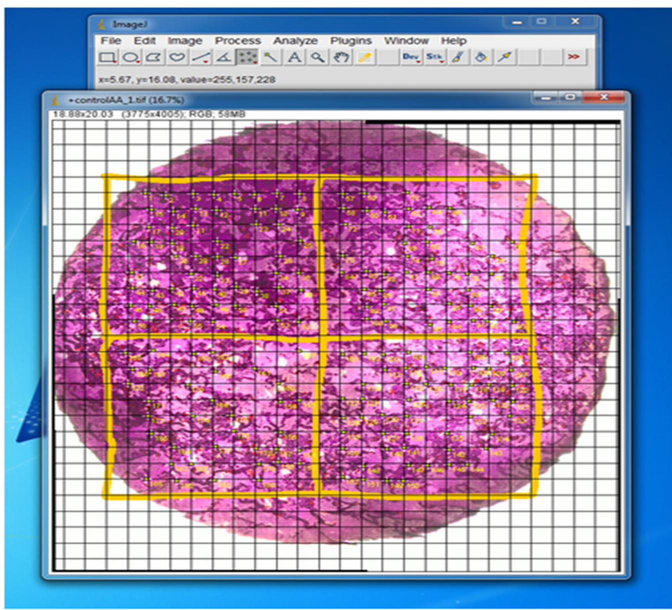

7. Click on the resorption pits intersecting points in all four 10x10 grids. Divide the final count by four to get the relative percentage resorption.

3.3.3 Cell morphology - Polarization-optical differential interference contrast (PlasDIC)

Following trypan blue exclusion, cells were seeded at 130 000 cells/cm^2 on glass coverslips in sterile 96-well plates with differentiation factors, RANKL (30ng/ml) and M-CSF (25ng/ml). Cells were exposed to LCPUFAs at 40µM for both differentiating osteoclast and mature osteoclast experiments. Culture media, differentiation factors and LCPUFAs were replaced every 2-3 days. The experiments were terminated 7 days after the onset of resorption.

PlasDIC was used to observe morphological characteristics of CD14+ monocytes and osteoclasts derived from these monocytes during exposure to the LCPUFAs. PlasDIC is an improved differential interference contrast method which provides high-quality imaging of cells grown in plastic cell culture containers. PlasDIC images were obtained with a Zeiss Axiocam MRc5 camera attached to a Zeiss Axiovert40 CFL microscope at 40x magnification (Zeiss, Göttingen, Germany).

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3.3.4 Visualization of actin ring, vitronectin receptor (VNR) and calcitonin receptor (CTR) expression

Following trypan blue exclusion, cells were seeded at 130 000 cells/cm^2 in duplicate on glass coverslips in sterile 24-well plates with differentiation factors, RANKL (30ng/ml) and M-CSF (25ng/ml). Cells were exposed to LCPUFAs at 40µM for both differentiating osteoclasts and mature osteoclasts. Culture media, differentiation factors and LCPUFAs were replaced every 2-3 days. The experiments were terminated 7 days after the onset of resorption.

Osteoclasts require complete actin rings as part of the sealing zone to enable them to resorb bone effectively. [202] Actin rings, vitronectin receptor (VNR) and calcitonin receptor (CTR) are considered markers of mature resorbing osteoclasts as they are expressed at the late stages of osteoclast differentiation. At the end of the culture period, cells were washed twice with PBS and fixed with 3.7% (v/v) formaldehyde in PBS for 15mins. The cells were then permeabilised for 5 min with 0.1% Triton X-100 and stained for actin with 5U/ml Alexa Fluor 568-Phalloidin (Life Technologies Carlsbad, CA, USA), for nucleus with 35µg/ml Hoechst (Sigma-Aldrich Inc., St Louis, MO, USA) and for VNR and CTR with 50µg/ml mouse anti-VNR and anti-CTR (R&D Systems, Minneapolis, MN, USA), respectively. Detection of anti-VNR and anti-CTR was performed with 2µg/ml Alexa Fluor 488-goat anti-mouse antibody (Life Technologies Carlsbad, CA, USA) and confocal laser scanning microscopy using a Zeiss Axiovert40 CFL microscope (Zeiss, Göttingen, Germany). [202]

3.3.5 Western blotting for cathepsin K, tartrate-resistant acid phosphatase (TRAP) and matrix metalloproteinase-9 (MMP-9)

Following trypan blue exclusion, cells were seeded at 130 000 cells/cm^2 in duplicate on glass coverslips in sterile 24-well plates with differentiation factors, RANKL (30ng/ml) and M-CSF (25ng/ml). Cells were exposed to LCPUFAs at 40µM for both differentiating

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osteoclasts and mature osteoclasts. Culture media, differentiation factors and LCPUFAs were replaced every 2-3 days. The experiments were terminated 7 days after the onset of resorption.

Cathepsin K, TRAP and MMP-9 are osteoclast-derived enzymes responsible for the degradation of the bone matrix. [200] At the end of culture the cells were washed in ice-cold PBS before lysis with Novex® Cell Extraction Buffer (Life Technologies, Carlsbad, CA, USA). The lysate was centrifuged at 13 000xg for 30mins using a Jouan Br4i centrifuge (DJB Labcare, Buckinghamshire, England) at 4°C for the removal of non-lysed fragments. Purified protein was quantified using a BCA protein determination kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Thereafter 20µg protein of each sample was loaded in sample buffer containing 1% β-mercaptoethanol and then run on a 4–12% NuPAGE Novex Bis-Tris precasted polyacrylamide gel (Life Technologies, Carlsbad, CA, USA), and subsequently electrotransferred onto a nitrocellulose membrane using an iBlot dry blotting system (Life Technologies, Carlsbad, CA, USA). The membranes were blocked for non-specific binding by incubation at room temperature in 5% skim milk powder for an hour. This was followed by incubation with rabbit polyclonal primary antibodies (1:1000) for GAPDH, Cathepsin K, TRAP or MMP-9 overnight at 4°C. The membranes were then washed and incubated in the goat anti-rabbit alkaline-phosphatase conjugated secondary antibody (1:1000) while shaking for an hour. The membranes were washed again and the blots were developed using an iBlot Western Detection Chromogenic Kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The membranes were scanned using a flatbed scanner (Ricoh Aficio, Johannesburg, South Africa) and ImageJ was used to enhance contrast of the images. [201]

3.3.6 Gene expression of prominent markers of osteoclastogenesis and resorption

Following trypan blue exclusion, cells were seeded at 130 000 cells/cm^2 in duplicate on glass coverslips in sterile 24-well plates with differentiation factors, RANKL (30ng/ml)

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and M-CSF (25ng/ml). Cells were exposed to LCPUFAs at 40µM for both differentiating osteoclasts and mature osteoclasts. Culture media, differentiation factors and LCPUFAs were replaced every 2-3 days. The experiments were terminated 7 days after the onset of resorption.

PCR was done to determine whether LCPUFAs affect bone resorption through modulation of the expression of the genes listed in Table 3.3. All primers were synthesized by InqabaBiotec (Pretoria, South Africa). At the end of culture, cells were homogenized in 300µl of TRI Reagent® (Sigma-Aldrich Inc., St Louis, MO, USA), scraped and transferred to eppendorf tubes. The samples were incubated with 60µl isopropyl alcohol for 10mins at room temperature and then centrifuged at 12 000xg for 10mins at 4°C using a Jouan Br4i centrifuge (DJB Labcare, Buckinghamshire, England). The supernatant was removed and the pellet was washed three times with 300µl 75% ethanol and then vortexed and centrifuged at 7500xg for 5 minutes with each wash. After allowing the pellet to air dry for 5mins it was resuspended in 20µl in nuclease-free water and RNA concentration was determined using the NanoDrop ND 1000 (Thermo Fischer Scientific, Waltham, MA, USA) at 260/280nm.

Equal concentrations of RNA were used for the synthesis cDNA. dNTPs (2.5mM), oligo (dT) primers (40µM), M-MuLV reverse transciptase (200units/µl) and nuclease-free water (up to 20µl) were added to each RNA sample. Samples were heated for 5mins at 65-80°C and then incubated for an hour at 42°C. Thereafter the enzyme was inactivated at 90°C for 10mins and the cDNA samples were stored at -20°C until needed.

For PCR reactions, a PxE 0.2 Thermal Cycler (Thermo Fischer Scientific, Waltham, MA, USA) was used. The reaction mixture contained 0.5µM of forward and reverse primer, 1µl template DNA, 1X KAPA2G Robust HotStart Ready Mix (KAPA biosystems, Cape Town, South Africa) and nuclease-free water (up to 25µl). Denaturation was at 95°C for 15 seconds, annealing was at 60°C for 15 seconds and extension was at 72°C for 15 seconds. The optimal number of cycles was 25 cycles for CTSK and MMP-9 and 35

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cycles for all other genes tested. PCR products were resolved on 1% agarose gel and visualized with ethidium bromide using a gel documentation system attached to a monochrome scientific grade camera (E- Box 1000/26M, Vilber Lourmat, Cedex, France).

Table 3.3: Summary of PCR primers

Gene	Forward Primer	Reverse Primer	No. of cycles
GAPDH	5' GATGACATCAAGAAGGTGGTGAAGC 3'	5'ATACCAGGAAATGAGCTTGACAAAG 3'	35
TRAP	5' CTTTGTAGCCGTGGGTGACT 3'	5'GGGAGCGGTCAGAGAATACG 3'	35
CTSK	5' CTGGAGGGCCAACTCAAGA 3'	5' CCTCTGCATTTAGCTGCCTT 3'	25
MMP9	5' GTCATCCAGTTTGGTGTCGCG 3'	5' AGGGGAAGACGCACAGCTC 3'	25
NFATc1	5' GTGGAGAAGCAGAGCAC 3'	5' ACGCTGGTACTGGCTTC 3'	35
c-Fos	5' CCCATCGCAGACCAGAGC 3'	5' ATCTTGCAGGCAGGTCGGT 3'	35
CA2	5' GAGTTTGATGACTCTCAGGACAA 3'	5' CATATTTGGTGTTCCAGTGAACCA 3'	35
DC-STAMP	5' ATGACTTGCAACCTAAGGGCAAAG 3'	5' GTCTGGTTCCAAGAAACAAGGTCAT 3'	35
RANK	5' TCTGCTTCTCTTCGCGTCTG 3'	5' CGTAGGGACCACCTCCTACA 3'	35

3.4 Statistics

3.4.1 Sample size

The primary objective was to compare the effects of AA and DHA on osteoclastogenesis and bone resorption in CD14+ monocytes. Four concentrations were considered (20, 40, 60 and 80µM) for each LCPUFA. Three replicate experiments were conducted with three different donors for each experiment, the outcomes of which were compared to the vehicle control (positive control). Results from representative experiments are shown. This study design and sample size had 16 degrees of freedom for error which, by convention, is acceptable.

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3.4.2 Data analysis

Data are representative of three independent experiments with three replicates, unless otherwise stated, in each experiment and was expressed as means and standard deviations. In some cases, the data was normalized to the control and/or expressed relative to the control. The data was analysed using appropriate two way analysis of variance (ANOVA) followed by Bonferroni post hoc test using GraphPad Prism software (GraphPad software Inc, California, USA). Testing was done at the 0.05 level of significance.

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CHAPTER 4

RESULTS

4.1 Effect of arachidonic acid and docosahexaenoic acid on osteoclast formation

4.1.1 Tartrate-resistant acid phosphatase activity in conditioned media

To determine if AA and DHA had an effect on TRAP released from the osteoclasts, a TRAP activity assay was done using conditioned media. TRAP is a lytic enzyme highly expressed by osteoclasts and can be used as a marker for osteoclast formation. Low levels of TRAP were found to be expressed in pre-osteoclasts. This level of TRAP remained constant in all concentrations until day 12 when resorption was first detected. From then there was a sharp increase in TRAP activity in the vehicle control and some other concentrations in both AA and DHA (Figure 4.1.1a).

It was found that AA significantly decreased TRAP activity in differentiating osteoclasts at 40-80µM from day 15. DHA in differentiating osteoclasts showed a significant decrease in TRAP activity only at 80µM from day 12 and at 60µM from day 15. AA showed inhibitory action on TRAP activity at lower concentrations than DHA in differentiating osteoclasts. (Figure 4.1.1a)

After the onset of resorption (day 12), the effects of AA and DHA were tested on TRAP activity in mature osteoclasts (Figure 4.1.1b). AA significantly decreased TRAP activity at 80µM from day 15 and at 40-80µM from day 17. DHA was shown to significantly decrease TRAP activity at 60-80µM in mature osteoclasts on day 15 but only at 80µM from day 17. Again AA was shown to have an inhibitory action at lower concentrations than DHA.

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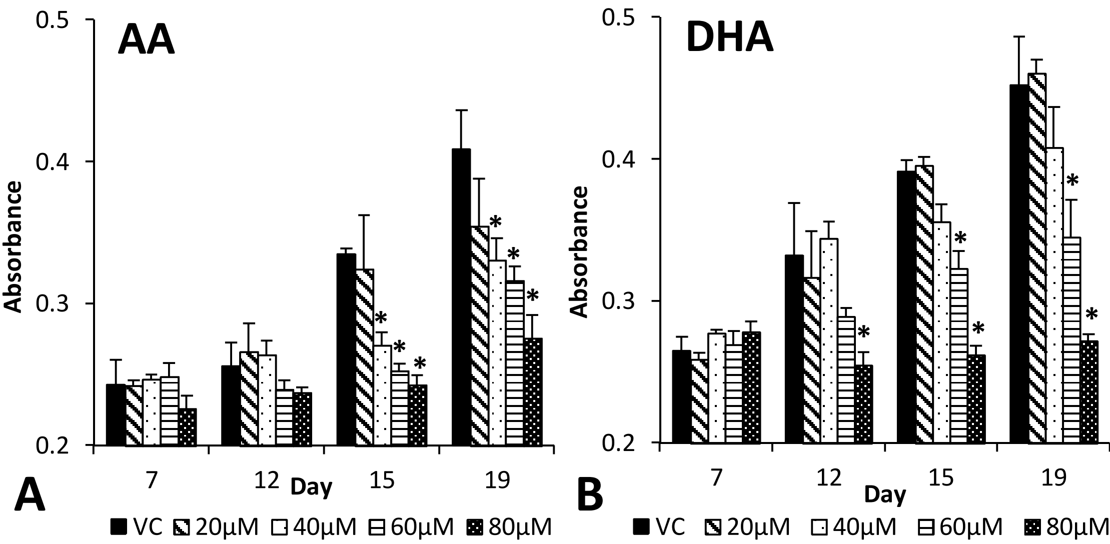

Figure 4.1.1a: Effect of AA and DHA on TRAP activity in differentiating osteoclasts. At medium changes, conditioned media were frozen and analysed for TRAP activity using para-nitrophenylphosphate (pNPP) as a substrate. TRAP activity was seen to start increasing from day 12-14, which coincides with the onset of resorption. This may show that the use of cells from the onset of resorption as mature osteoclasts is valid. The legend is displayed underneath the graphs. **A.** Cells exposed to AA in differentiating osteoclasts showed a decrease in TRAP activity at 40- 80µM from day 15. **B.** Cells exposed to DHA in differentiating osteoclasts only showed a significant decrease in TRAP activity at 80µM from day 12 and 60-80µM from day 15. ∗Significant difference from control (*P*<0.05). VC – Vehicle control.

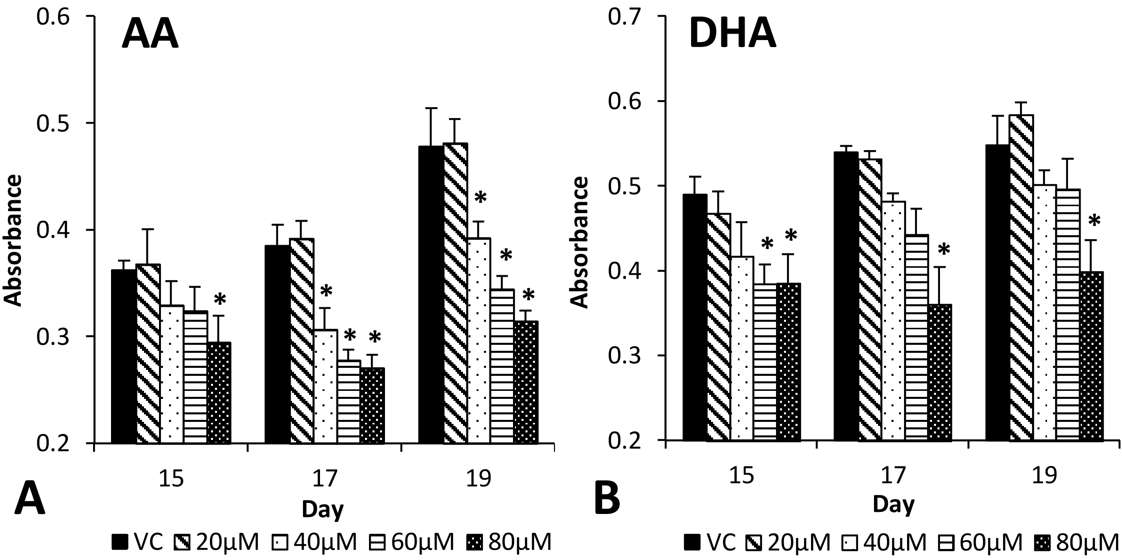

Figure 4.1.1b: Effect of AA and DHA on TRAP activity in mature osteoclasts. The principle and method of testing was the same as in 4.1.1a. The legend is displayed underneath the graphs. **A.** When mature osteoclasts were exposed to AA, a decrease in TRAP activity was seen at 80µM from day 15 and at 40, 60 and 80µM from day 17. **B.** When DHA was added to mature osteoclasts, again a significant decrease in TRAP activity is seen at 60-80µM on day 15 but from day 17 only 80µM significantly decreased TRAP activity. ∗Significant difference from control (*P*<0.05). VC – Vehicle control.

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4.1.2 Tartrate-resistant acid phosphatase positive osteoclast numbers

AA was shown to affect the formation of TRAP+ osteoclasts in differentiating osteoclasts in a dose-dependent manner (Figure 4.1.2a). A significant decrease in osteoclastogenesis was seen at all the concentrations tested. Almost no osteoclasts were formed after differentiating osteoclasts were exposed to AA at 60 and 80µM. DHA showed inhibitory effects on osteoclastogenesis at 40-80µM in differentiating osteoclasts. Both LCPUFAs might have been harmful to differentiating osteoclasts at 60-80µM (Figure 4.1.2b). As both LCPUFAs at 40µM, showed no signs of being harmful but still showed inhibitory effects on osteoclast formation in differentiating osteoclasts, this concentration was chosen for further experiments. At 40µM AA showed a 60% reduction in osteoclast formation while DHA only showed a 20% reduction. AA again showed a much stronger inhibitory effect than DHA at the same concentration.

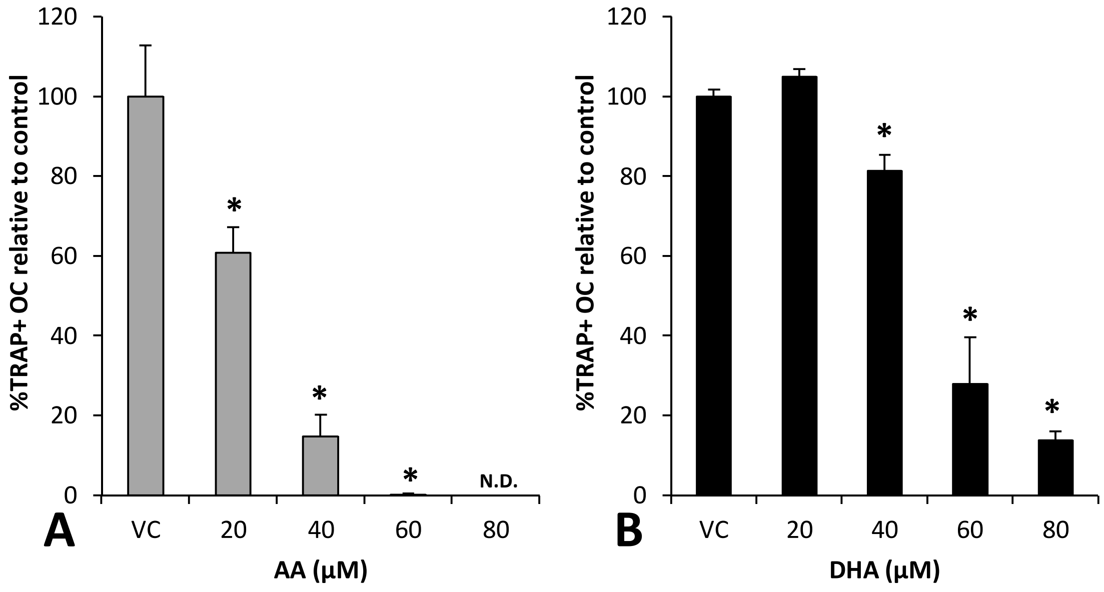

Figure 4.1.2a: Effect of AA and DHA on TRAP+ osteoclast numbers in differentiating osteoclasts. TRAP+ cells with 3 or more nuclei were counted as mature osteoclasts under a microscope. Results are expressed as a percentage relative to the control. **A.** Cells exposed to AA showed a significant decrease in the number of TRAP+ osteoclasts at all concentrations in differentiating osteoclasts. **B.** DHA significantly decreased the number of TRAP+ osteoclasts in differentiating osteoclasts at 40, 60 and 80µM. ∗Significant difference from control (P<0.05). VC – Vehicle control. OC – osteoclasts. N.D. – No osteoclasts detected.

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AA | Vehicle control | DHA

20µM
500 µm
40µM
500 µm
60µM
500 µm
80µM
500 µm

500 µm

20µM
500 µm
40µM
500 µm
60µM
500 µm
80µM
500 µm

Figure 4.1.2b: Effect of AA and DHA on TRAP+ osteoclast formation in differentiating osteoclasts (photomicrographs). Cells were seeded on glass coverslips and exposed to LCPUFAs for 21 days from day 3 and TRAP staining was performed. Many large osteoclasts are seen in the control. In cells exposed to AA, fewer TRAP+ osteoclasts (large red cells) are seen from 20µM compared to the control. In cells exposed to DHA, a decrease in osteoclast formation compared to the control can be seen from 40µM. For both LCPUFAs cell density appears lower at 60-80µM, indicating possible harmful effects at these concentrations.

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When the LCPUFAs were added to the mature osteoclasts, neither FA showed any effect on the number of TRAP+ osteoclasts at all concentrations tested (Figure 4.1.2c).

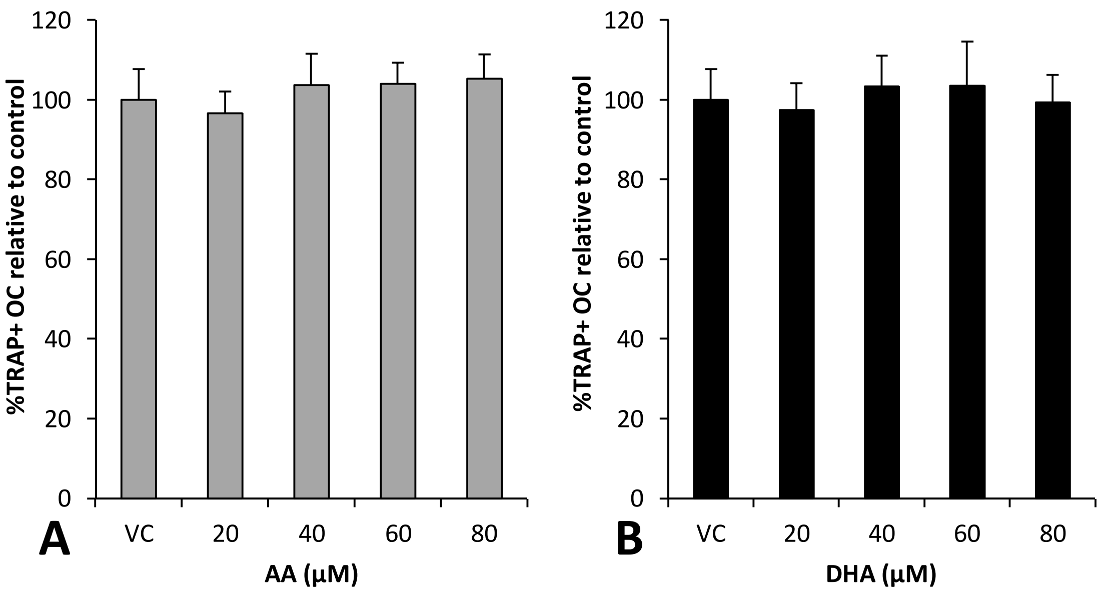

Figure 4.1.2c: Effect of AA and DHA on TRAP+ osteoclast numbers in mature osteoclasts. TRAP+ cells with 3 or more nuclei were counted as mature osteoclasts under a microscope. Results are expressed as a percentage relative to the control. Cells were exposed to LCPUFAs from the onset of resorption. **A and B.** Mature osteoclasts showed no significant change in osteoclast numbers when exposed to either AA or DHA at all the concentrations tested. VC – Vehicle control. OC – osteoclasts.

4.2 Effect of arachidonic acid and docosahexaenoic acid on cell morphology

PlasDIC photomicrographs were taken from day 7 of culture. At this stage, the cells in all the exposure conditions looked similar. In differentiating osteoclasts exposed to either LCPUFA, multinucleated cells are only present from day 14 (Figure 4.2a). These cells are also much smaller and have fewer nuclei than the osteoclasts of the control. By day 21, a few more osteoclasts can be seen in the cells exposed to DHA compared to those exposed to AA at 40µM. By day 12, small multinucleated cells can be seen in the vehicle control and in the mature osteoclasts groups (Figure 4.2b). After the mature osteoclasts are exposed to LCPUFAs on day 14, large multinucleated cells morphologically similar to those of the control are still formed.

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	Vehicle control	40µM AA Diff OC	40µM DHA Diff OC
Day 7	100 µm	100 µm	100 µm
Day 12	100 µm	100 µm	100 µm
Day 14	100 µm	100 µm	100 µm
Day 17	100 µm	100 µm	100 µm
Day 19	100 µm	100 µm	100 µm
Day 21	100 µm	100 µm	100 µm

Figure 4.2a: Effect of AA and DHA on cell morphology in differentiating osteoclasts. Fewer and smaller osteoclasts with 3 or more nuclei are seen when compared to the vehicle control. A few more osteoclasts can be seen in the cells exposed to DHA compared to those exposed to AA. Large multinucleated osteoclasts indicated with black arrows.

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	Vehicle control	40µM AA Mat OC	40µM DHA Mat OC
Day 7			
Day 12			
Day 14			
Day 17			
Day 19			
Day 21			

Figure 4.2b: Effect of AA and DHA on cell morphology in mature osteoclasts. Multinucleated cells can be seen in the vehicle control and mature osteoclasts experiments from day 12. Mature osteoclasts were exposed to LCPUFAs from the onset of resorption (day 14). These cells appear to grow similarly to the RANKL only control and no morphological difference is seen between them and the control after 21 days. Large multinucleated osteoclasts indicated with black arrows.

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4.3 Effect of arachidonic acid and docosahexaenoic acid on resorption pit formation

To analyse whether AA and DHA have any effects on mature osteoclasts, cells were seeded on dentine discs and resorption assays were conducted. As both LCPUFAs were already shown to decrease the formation of osteoclasts in differentiating osteoclasts, resorption assays were only conducted on mature osteoclasts. Neither LCPUFA showed a significant change in resorption at 20µM (Figure 4.3a). Compared to the control, both LCPUFAs were shown to decrease resorption in mature osteoclasts from 40-80µM as can be seen on the dentine discs (Figure 4.3b). At 40µM both LCPUFAs showed a decrease of about 50% in resorption. However, at 60-80µM AA showed a stronger inhibitory effect on resorption than DHA.

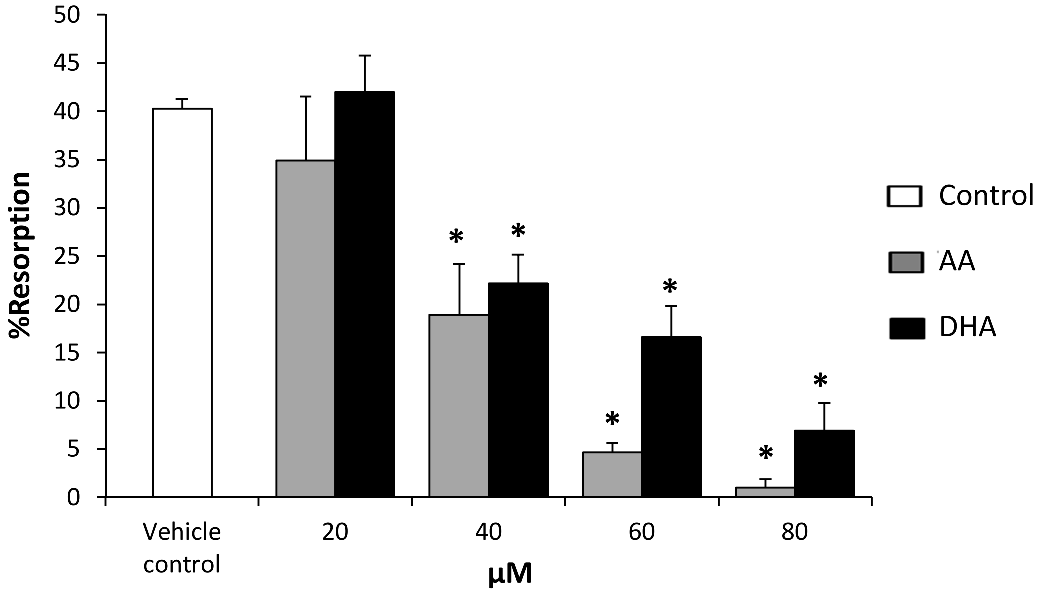

Figure 4.3a: Effect of AA and DHA on resorption pit formation. Resorption was determined using an indirect method known as point counting. Resorption assays were only conducted on mature osteoclasts. Both AA and DHA had an inhibitory effect on resorption from 40-80µM. At 40µM the reduction in osteoclastic resorption of both LCPUFAs was about 50%. At 60 and 80µM AA had a stronger inhibitory effect on resorption than DHA. *Significant difference from control (*P*<0.05).

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AA | Vehicle control | DHA

20µM | | 20µM
40µM | | 40µM
60µM | | 60µM
80µM | | 80µM

Figure 4.3b: Stained entire dentine discs. Resorption pits are the dark purple trails. The red dots are mature TRAP stained osteoclasts. At 20µM for both LCPUFAs, resorption pit formation appears similar to that of control. From 40-80µM a clear decrease is seen in the number of resorption pits. The number of osteoclasts remains fairly constant.

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4.4 Effect of arachidonic acid and docosahexaenoic acid on of actin ring formation and vitronectin receptor (VNR) and calcitonin receptor (CTR) expression

After 21 days of culture, cells were fluorescence stained for actin, VNR and CTR. VNR and CTR are cell surface receptors that are highly expressed in mature osteoclasts. Alexa Fluor 568-Phalloidin is an actin probe that stains the actin rings of the osteoclasts red. Hoechst is a blue nuclear stain while Alexa Fluor 488-goat anti-mouse antibody will bind to anti-VNR and anti-CTR and stain green. The integrity of the actin rings and the presence of VNR and CTR were analysed qualitatively.

Although fewer osteoclasts were seen in the differentiating osteoclasts exposed to either LCPUFA at 40µM, when compared to the control, neither LCPUFA showed any effect on the integrity of the actin rings formed. This may indicate that neither LCPUFA had a harmful effect on the osteoclasts at this concentration. AA showed a slight inhibition of VNR but greatly reduced the expression of CTR in differentiating osteoclasts. DHA slightly inhibited VNR expression, but had no effect on CTR expression in differentiating osteoclasts. (Figure 4.4a)

In mature osteoclasts, the osteoclasts formed resembled those in the control. Neither LCPUFA showed an effect on the integrity of the actin rings when compared to the control in mature osteoclasts. Both LCPUFAs showed a slight inhibition of VNR and CTR in mature osteoclasts. (Figure 4.4b)

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Actin | VNR | Overlay | Actin | CTR | Overlay

No RANKL

Vehicle control

40µM AA Diff OC

40µM DHA Diff OC

Figure 4.4a: Effect of AA and DHA on actin ring formation and VNR and CTR expression in differentiating osteoclasts. After 21 days of culture, cells were fixed and stained with phalloidin for actin rings, and either anti-VNR or anti-CTR. No large actin rings or positive stains for VNR or CTR are seen when no RANKL is added. Many large multinucleated osteoclasts, positive for both VNR and CTR, were seen in the vehicle controls. When the cells were exposed to the LCPUFAs, fewer actin rings were seen when compared to the control. DHA affected the expression of VNR, while AA seemed to slightly reduce VNR expression and greatly reduce the expression of CTR. Red – Actin. Blue – Nuclei. Green – VNR or CTR.

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Actin VNR Overlay Actin CTR Overlay

No RANKL

Vehicle control

40µM AA Mat OC

40µM DHA Mat OC

Figure 4.4b: Effect of AA and DHA on actin ring formation and VNR and CTR expression in mature osteoclasts. After 21 days of culture, cells were fixed and stained with phalloidin for actin rings, and either anti-VNR or anti-CTR. No large actin rings or positive stains for VNR or CTR are seen when no RANKL is added. Many large multinucleated osteoclasts, positive for both VNR and CTR, were seen in the vehicle controls. Slight decreases in VNR and CTR are seen in mature osteoclasts exposed to either LCPUFA, with AA showing greater effects. Red – Actin. Blue – Nuclei. Green – VNR or CTR.

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4.5 Effect of arachidonic acid and docosahexaenoic acid on osteoclast specific proteins

As resorption was shown to be decreased, the expression of resorption enzymes (cathepsin K, TRAP and MMP-9) were analysed by western blot. A decrease in the expression of these enzymes could explain the decrease in resorption witnessed in this study.

Both AA and DHA were shown to decrease the expression of cathepsin K, TRAP and MMP-9 in differentiating osteoclasts (Figure 4.5a). The analysis of the bands indicated that AA and DHA had similar reduction in cathepsin K expression (Figure 4.5b). AA had a significantly greater inhibitory effect than DHA on TRAP and MMP-9 expression in differentiating osteoclasts.

Both AA and DHA were shown to decrease the expression of cathepsin K in mature osteoclasts (Figure 4.5a). AA showed an inhibitory effect of TRAP and MMP-9 expression while DHA showed no effect on either of these enzymes (Figure 4.5b). Interestingly, the level of MMP-9 expression in cells receiving no RANKL was high in comparison to cells exposed to either LCPUFA and receiving RANKL.

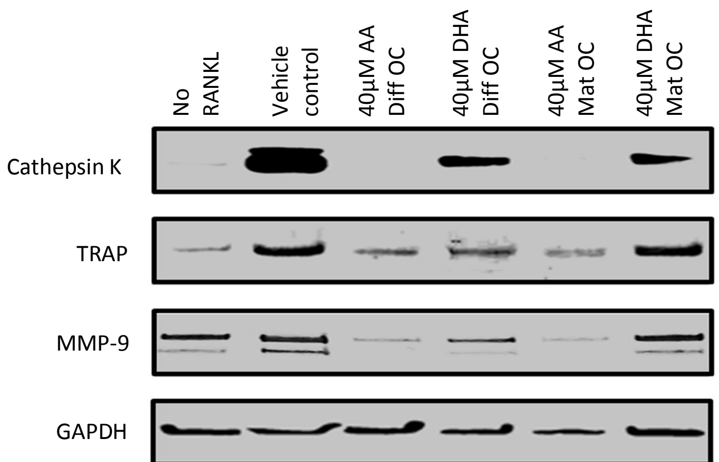

Figure 4.5a: Effect of AA and DHA on resorption enzymes (bands). The thickness of the bands corresponds to the relative expression of the protein. GAPDH was used as a loading control. Compared to the control, cathepsin K expression is decreased in both differentiating osteoclasts and mature osteoclasts by both LCPUFAs. TRAP and MMP-9 show no change in mature osteoclasts exposed to DHA.

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Figure 4.5b: Effect of AA and DHA on resorption enzymes. Both fatty acids were shown to decrease the expression of cathepsin K, TRAP and MMP-9 in both differentiating osteoclasts. Mature osteoclasts exposed to DHA showed no significant decrease in TRAP and MMP-9 expression. However a significant decrease in TRAP and MMP-9 was seen in mature osteoclasts exposed to AA. Relative densities were determined using ImageJ software. *Significant difference from control ($P<0.05$).

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4.6 Effect of arachidonic acid and docosahexaenoic acid on osteoclast specific genes

As both LCPUFAs were shown to affect the expression of the resorption enzymes (cathepsin K, TRAP and MMP-9), genes involved in the effects of RANK signalling in osteoclasts were analysed by PCR to ascertain whether the LCPUFAs exert their effects by targeting the RANK signalling pathway.

In differentiating osteoclasts, all the genes tested showed a significant reduction compared to the control when exposed to either LCPUFA at 40µM (Figure 4.6a). Some correlation was seen between the expression of proteins cathepsin K, TRAP and MMP-9, as assessed by the western blot, and the expression of their genes. AA showed a stronger inhibitory effect than DHA on the expression of MMP9, c-Fos, RANK and CA2 in differentiating osteoclasts (Figure 4.6b).

All the genes tested showed a significant reduction when exposed to either LCPUFA in mature osteoclasts, except CA2 and TRAP exposed to DHA where no visible changes were seen (Figure 4.6b). However, DHA showed a stronger inhibitory effect than AA on the expression of c-Fos and RANK. Interestingly, DHA showed no effect on the expression of the MMP-9 protein in mature osteoclasts but a significant decrease was shown in MMP9 gene expression. No change was seen in TRAP protein expression in mature osteoclasts exposed to DHA and this was correlated with no change in gene expression of TRAP (Figure 4.6b).

Elevated levels of MMP9 were seen in undifferentiated monocytes receiving no RANKL. Although protein levels of MMP-9 were significantly lower in cells receiving no RANKL, gene levels of MMP9 showed no significant change (Figure 4.6a).

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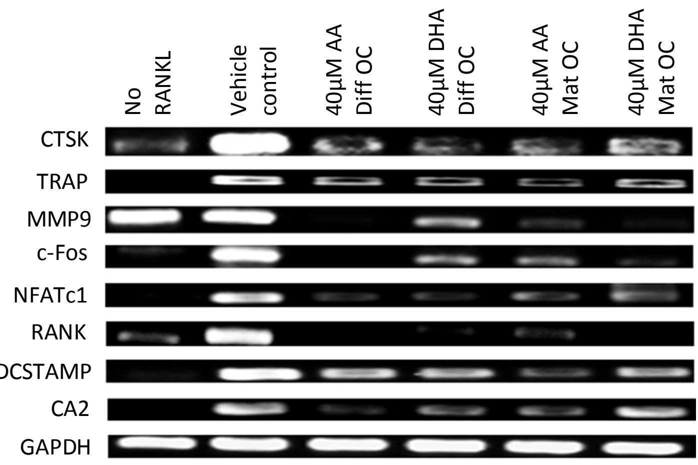

Figure 4.6a: Effect of AA and DHA on gene expression (bands). The thickness of the bands corresponds to the relative expression of the gene. GAPDH was used as a loading control. Compared to the control, the expression of all the genes tested was decreased in both differentiating and mature osteoclasts by both LCPUFAs with two exceptions. TRAP and CA2 show no change in mature osteoclasts exposed to DHA.

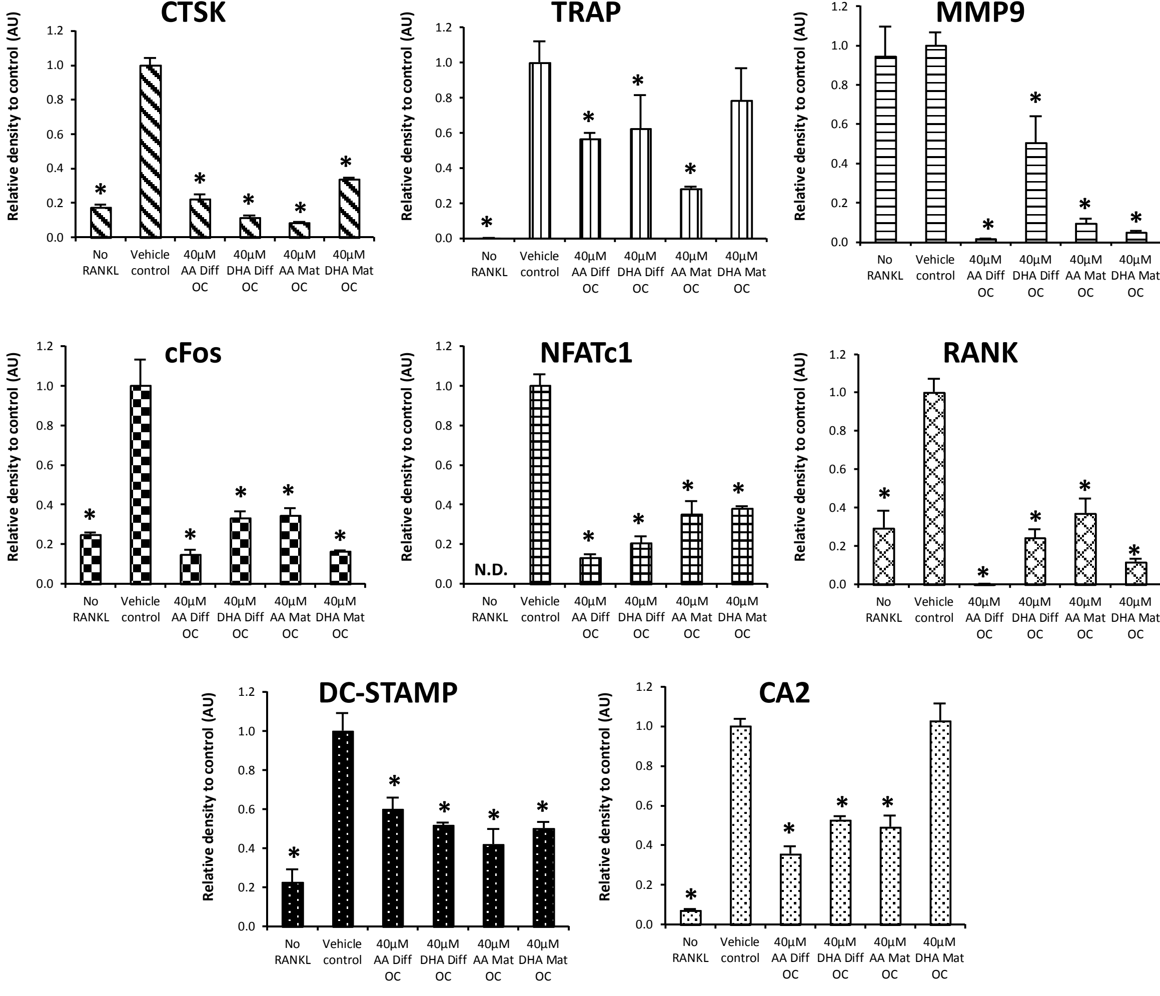

Figure 4.6b: Effect of AA and DHA on gene expression. Gene expression was shown to decrease in differentiating and mature osteoclasts when exposed to either LCPUFA in all the genes tested, except with CA2 and TRAP in mature cells exposed to DHA where no change is seen. High levels of expression of MMP9 were seen in undifferentiated cells which correlate with the results in the western blot. Relative densities were determined using ImageJ software and graphs were drawn for each gene tested. *Significant difference from control (P<0.05). N.D. – Not detected.

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CHAPTER 5

DISCUSSION

In the present study, the effects of an ω-3 LCPUFA, DHA, and an ω-6 LCPUFA, AA, on osteoclast formation, resorption, cell morphology, actin ring formation, VNR and CTR expression, protein expression and gene expression were investigated in human CD14+ monocytes during differentiation and after maturation into osteoclasts. The purpose of the study was to reveal possible mechanisms of action of AA and DHA in human osteoclasts as no such studies have been reported in a human osteoclast cell line before. As evidenced by the results in chapter 4, both AA and DHA can affect osteoclast formation and activity *in vitro* in the human CD14+ osteoclast cell line. In this chapter each of the results will be discussed in detail and their relevance will be explained. These results will be compared to current knowledge of the effects of AA and DHA in animal cell lines. Possible mechanisms of action of AA and DHA in human CD14+ monocytes will then be discussed. This chapter will conclude with recommendations for further research.

5.1 Overview of findings

In order to test the effects of AA and DHA on RANKL-induced osteoclast formation, CD14+ monocytes were exposed to the LCPUFAs from the beginning of culture (differentiating osteoclasts) or from the onset of resorption (mature osteoclasts). TRAP is an enzyme highly expressed by osteoclasts and can be used as a marker for osteoclast formation. TRAP activity in the medium is an indirect method of determining the quantity of the mature osteoclasts. TRAP activity levels were decreased by both LCPUFAs in differentiating osteoclasts and mature osteoclasts. However, these results did not always correlate with the cell count that was carried out. Some correlation was seen between TRAP activity and resorption. As TRAP can be

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expressed by pre-fused osteoclasts, TRAP activity results may not be very sensitive to changes in the number of osteoclasts formed (See Figure 5.1). [200, 203] In differentiating osteoclasts there was some correlation between the TRAP assay results and the western blot results for TRAP expression when exposed to either LCPUFA at 40µM: the assay only showed a significant decrease when exposed to AA and the western blot showed a significant decrease when exposed to either LCPUFA. However in mature osteoclasts, AA showed decreases at 40µM in TRAP levels as well as the western blot for TRAP, while DHA at 40µM showed no change in both experiments. This is interesting as the TRAP assay takes into account only TRAP that has been released into the media whereas the western blot measures intracellular TRAP levels. In this study the assay was found to only be sensitive to large changes in osteoclast formation and function. Interestingly, TRAP activity was shown to increase exponentially from the onset of resorption, showing that the TRAP may have potential as a marker for resorption, *in vitro*. This may indicate that TRAP plays a vital role in resorption by osteoclasts. It is believed that TRAP is involved in the formation of ROS needed to resorb the inorganic component of bone. [13]

TRAP positive cells with three or more nuclei were counted as mature osteoclasts as has been reported previously. [35, 200] Differentiating osteoclasts exposed to AA showed a decrease in the number of mature osteoclasts at all concentrations tested, while differentiating osteoclasts exposed to DHA showed a decrease in mature osteoclasts from 40-80µM. AA showed a stronger inhibitory effect on osteoclastogenesis than DHA. At 40µM for both LCPUFAs, osteoclast formation was decreased but cell density was maintained. However, at 60-80µM cell density was reduced indicating that these concentrations may affect cell numbers in differentiating osteoclasts.

In mature osteoclasts, no effect was seen on the number of TRAP positive osteoclasts when exposed to either LCPUFA at all concentrations tested compared to the vehicle control. Interestingly, at 60-80µM neither LCPUFA affected osteoclast numbers in mature osteoclasts. This could indicate that early differentiating osteoclasts are more susceptible to the effects of the LCPUFAs than late stage bone resorbing osteoclasts.

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Furthermore, this may indicate that the LCPUFAs have no detrimental effects on already matured osteoclasts. As stated earlier, osteoclasts and osteoblasts are coupled together in the bone remodelling cycle (See 2.1.3). Therefore the presence of osteoclasts is required for osteoblast function. If LCPUFAs decrease osteoclast activity without affecting the number of mature osteoclast, the balance between these two cells required for bone remodelling could be maintained.

As the LCPUFAs tested did not affect osteoclast numbers in mature osteoclasts, resorption was analysed in order to test whether osteoclast function was affected by these LCPUFAs. In differentiating osteoclasts, resorption was not analysed because cell numbers were shown to decrease and therefore resorption would be expected to decrease as well, and the preparation of the dentine slices is cumbersome and costly and therefore they were only used where it was deemed necessary. Exposure of matured osteoclasts to either LCPUFAs was seen to decrease resorption at 40-80µM. At 40µM, AA and DHA showed similar inhibitory effects on resorption. However, from 60-80µM, AA had a stronger inhibitory effect than DHA at equal concentrations. This may indicate that AA has a stronger inhibitory effect on osteoclastic resorption than DHA. It is important to keep in mind that this is an *in vitro* study, and this finding does not necessarily mean that AA is more beneficial to bone health than DHA. Other factors, such as the effect of the LCPUFAs in other bone cells, should be evaluated before a conclusion can be made on which LCPUFA is more beneficial. However, as previously stated, the primary function of osteoclasts is bone resorption so it is interesting to note that this function of osteoclasts was decreased without causing a decline in osteoclast numbers. Therefore it could be speculated that the LCPUFAs can affect mature osteoclast activity through modulating gene and protein expression.

The lowest effective concentration for both LCPUFAs on osteoclast formation in differentiating osteoclasts and resorption pit formation in mature osteoclasts was 40µM. Therefore this concentration was used for all subsequent experiments. As the LCPUFAs at 60-80µM could show harmful effects in differentiating osteoclasts, these concentrations were not considered for further experiments

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Markers	CD14+ monocyte	Prefused osteoclast	Mature/ multicellular osteoclast	Bone resorbing osteoclast
CD14	+	+		
c-Fms	+	+	+	+
RANK		+	+	+
c-Fos		+	+	+
NFATc1		+	+	+
TRAP		+	+	+
DC-STAMP		+	+	+
CTR			+	+
VNR			+	+
MMP-9			+	+
Cathepsin K			+	+
CA2			+	+
Actin Ring				+
Ruffled border				+
Bone resorption				+

Figure 5.1: Expression of osteoclast markers. The figure indicates when the expression of certain markers of osteoclast differentiation and function can be expected to be detected. **+** indicates that the marker is present at this stage of differentiation. Image created with information from Sørensen *et al*. (2007) and Arboleya *et al*. (2014). [200, 203]

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PlasDIC photomicrographs were taken to analyse whether any morphological changes could be observed in osteoclasts when exposed to the LCPUFAs. In mature osteoclasts, no clear morphological differences could be seen between the osteoclasts exposed to the LCPUFAs and the control osteoclasts. The shape, size and number of nuclei appear similar to those in the control. That there is no effect on cell morphology could further suggest that the LCPUFAs have no harmful effects on mature osteoclasts.

In differentiating osteoclasts, fewer osteoclasts were seen and these osteoclasts were smaller and had fewer nuclei than those of the control. However, many undifferentiated monocytes were still present. Fewer and smaller osteoclasts could indicate that the LCPUFAs delay the fusion of precursors and therefore delay the formation of osteoclasts and not prevent it. The presence of many undifferentiated cells could indicate that exposing the osteoclasts to LCPUFAs at these concentrations had no harmful effects on the cells.

Actin rings are a dense belt of actin that forms around the cells, that maintains the structural integrity of the osteoclast and is thought to play a role, along with VNR (integrin $\alpha_v\beta_3$), in the formation of the sealing zone. [6] CTR is the receptor for calcitonin, a hormone that decreases blood calcium levels by inhibiting osteoclast formation and bone resorption. [24] CTR is highly expressed on mature osteoclast to ensure blood calcium levels can be maintained. Actin rings, VNR and CTR can therefore be used as markers for mature functional osteoclasts.

Actin ring formation, VNR and CTR expression were analysed by qualitative assessment. Although fewer and smaller actin rings could be seen in differentiating osteoclasts exposed to either LCPUFA, there was no visible damage to the actin ring structure as such. However VNR and CTR expression seemed to be decreased in differentiating osteoclasts exposed to AA. DHA showed normal expressions of CTR but decreased expression of VNR in differentiating osteoclasts, which could indicate that DHA had milder effects on osteoclast formation.

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In mature osteoclasts no difference was observed in actin ring formation when exposed to either LCPUFA. DHA and AA slightly inhibited CTR expression. Both LCPUFAs were also shown to decrease VNR expression, with AA seemingly showing greater inhibition, which may explain how the LCPUFAs decreased bone resorption as VNR is needed for the osteoclast to attach to bone. The stronger inhibitory effect of AA on VNR expression could also explain why AA has a stronger inhibitory effect on resorption than DHA.

As the LCPUFAs tested were shown to affect resorption, the expression of resorptive enzymes were analysed through a western blot. Cathepsin K, TRAP and MMP-9 are all proteins that play a role in resorption. [200] Cathepsins play a role in the breakdown of bone collagen and other organic components of bone. [204] Of the known cathepsins, cathepsin K is the most abundantly expressed in humans and high levels are found in the resorption lacunae. Therefore it could be of vital importance in osteoclastic resorption. [205] Human patients with a mutation in the cathepsin K gene are known to develop pycnodystosis, a condition of abnormally dense bones. [206] Cathepsin K and the degraded products are endocytosed into the osteoclast where they fuse with vesicles containing TRAP. [36] TRAP is then cleaved into an activated form by cathepsin K. [36] High levels of MMP-9 are also found in the resorption lacunae. [6] However, MMP-9 deficient mice only show brief disruptions in bone resorption, indicating that cathepsin K may be more vital for resorption than MMP-9. [207]

Cathepsin K levels were seen to be decreased by both LCPUFAs in differentiating osteoclasts and mature osteoclasts when compared to the control. As osteoclastogenesis was decreased in differentiating osteoclasts, lower cathepsin K levels were expected. However, the lower levels of cathepsin K could explain the decrease in resorption by mature osteoclasts exposed to the LCPUFAs. Only mature osteoclasts exposed to DHA did not cause a significant reduction in TRAP and MMP-9 expression. The higher concentrations of TRAP and MMP-9 could explain why DHA had a lesser effect on resorption than AA. Interestingly, cells grown in the absence of RANKL still expressed high levels of MMP-9 similar to that of the control even though

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no osteoclasts were present. However, as Lepidi *et al*. have established, human monocytes in culture (not receiving any RANKL) will differentiate into macrophages and produce high levels of MMP-9 which may explain our observations in the cells receiving no RANKL. [208]

Cathepsin K, TRAP and MMP-9 are products of RANK signalling (See Figure 2.3). Therefore genes involved in the RANK signalling cascade were analysed by PCR to determine whether the LCPUFAs tested have effects on this crucial pathway. The eight genes tested all play a role in osteoclast function or formation. CTSK encodes the enzyme cathepsin K while TRAP and MMP9 encode for the enzymes TRAP and MMP-9 respectively. In the case of TRAP, there was no significant change in gene expression in mature osteoclasts exposed to DHA which correlates to the results seen in the western blot. A decrease in expression of genes is associated with a decrease in expression of the proteins they encode in all but one case. In mature osteoclasts exposed to DHA, a significant decrease is seen in MMP9 gene expression while no significant change occurred in protein expression. As there are many steps between gene transcription and translation, it is difficult to determine the cause of this poor correlation between MMP9 gene expression and protein expression in mature osteoclasts exposed to DHA. The different half-lives of proteins and genes may also mean that changes in mRNA expression will not lead to an immediate detectable change in protein expression. This poor correlation between gene and protein could indicate that DHA has less aggressive effects on osteoclasts than AA. As seen in previous results in our study, the inhibitory effect of AA is generally greater than that of DHA.

As discussed previously, c-Fos and NFATc1 play a role in the RANK signalling cascade (See Figure 2.3). c-Fos activates NFATc1, which is known as the master regulator of osteoclasts. [29] Both LCPUFAs decreased the expression of c-Fos and NFATc1 in differentiating osteoclasts and mature osteoclasts. As c-Fos is upstream of NFATc1, it could be speculated that the decrease in c-Fos is the cause of the decrease in NFATc1. A decrease in c-Fos expression could indicate that the LCPUFAs target upstream proteins such as JNK1, ERK1 or NF-κB which are known to activate c-Fos during RANKL-

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induced osteoclastogenesis. DC-STAMP, which plays a role in cell-to-cell fusion and is downstream of NFATc1, [30] was also down-regulated by both LCPUFAs in differentiating osteoclasts and mature osteoclasts. By down-regulating DC-STAMP, the fusion of pre-osteoclasts would be prevented and this could explain why fewer osteoclasts are seen in differentiating osteoclasts exposed to either LCPUFA.

Mature osteoclasts exposed to DHA showed no effect on CA2, which plays a role in acidification of the resorption lacunae and is downstream of NFATc1. An acidic environment is required for the function of the enzymes cathepsin K and MMP-9. Although cathepsin K expression was similar in mature osteoclasts exposed to either LCPUFA, the higher expression of CA2 (coupled with higher levels of MMP-9 expression) in cells exposed to DHA may be the reason DHA showed lesser effects on resorption than AA.

The RANK gene was also shown to be down-regulated by both LCPUFAs. As RANK expression is increased through a pathway directed by the binding of M-CSF to c-Fms, this could mean that LCPUFAs can affect this pathway as well. By decreasing the RANK expression, fewer RANK receptors would be available for binding with RANKL thereby decreasing osteoclast formation and function.

5.2 Integration with current knowledge

Current studies in animal cell lines have shown that LCPUFAs can affect osteoclast formation. These studies were conducted in differentiating osteoclasts as the cells were exposed to LCPUFAs before they reached maturation. Rahman *et al.* showed that DHA (50µM) in RAW 264.7 murine macrophages inhibited osteoclast formation, TRAP activity, the expression of the genes MMP9, TRAP, CTSK and CTR, and the expression of the c-Fos protein. [130] Yuan *et al*. found that DHA (10µM) in murine BMMs inhibited osteoclast formation and the expression of the genes NFATc1, DC-STAMP and TRAP. [129] Our findings also suggest that DHA may have similar effects in human osteoclasts as we also recorded decreases in osteoclast formation as well as decreases in CTSK, TRAP,

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MMP9, c-Fos, NFATc1 and DC-STAMP gene expression. Although, Yuan *et al*. found that AA (10µM) enhanced osteoclastogenesis, [129] Cornish *et al.* showed that AA decreased osteoclast formation at 2.5µg/ml (≈ 7.5µM) in murine BMMs, [134] similar to the effects of AA we observed. The different concentrations of RANKL used in the two studies may explain these contradictory results. Boeyens *et al*. have shown that AA (20µg/ml (65µM)) and DHA (20µg/ml (60µM)) has similar effects to what has been previously published in RAW 264.7 macrophages, but also reported that actin rings appeared fewer and smaller when exposed to AA, [137] similar to our findings. Interestingly, this study found that DHA had a stronger inhibitory effect on osteoclast formation than AA. [137] We have reported that AA showed a stronger inhibitory effect than DHA in the present study. However, our study was conducted in a human primary cell model and therefore gives more precise information on the mechanisms that could occur in humans.

There are currently no published studies on the effects of LCPUFAs on already matured bone-resorbing human osteoclasts. This study is the first to show that LCPUFAs can affect mature human osteoclasts as well as differentiating osteoclasts. It was also shown that the LCPUFAs tested showed no harmful effects on mature osteoclasts as osteoclast numbers were unaffected. The inhibitory effect on resorption can be said to have occurred by affecting osteoclast genes without causing harmful effects on the osteoclast themselves. Therefore, we hypothesize that reduced osteoclast activity in mature osteoclasts and reduced formation in differentiating osteoclasts by modulating osteoclast-specific genes may be a mechanism by which LCPUFAs prevent bone loss.

5.3 Possible mechanisms of action of AA and DHA in human CD14+ monocytes

Based on our findings some speculation can be made about the mechanisms of action of AA and DHA. Whitcomb *et al*. demonstrated that LCPUFAs can be incorporated into the cell membranes and affect membrane viscosity of human adrenocortical cells, *in vitro*. [209] As stated earlier, the increase in ω-3 LCPUFAs in the membrane can affect

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membrane proteins such as TLR4 which can down-regulate NF-κB, a crucial stimulator of osteoclast formation and function. [93] It is possible that in this study, the LCPUFAs exerted their effects through incorporation into the cell membrane. [93]

Binding of TNF-α to its receptor (TNFR) can stimulate osteoclast formation through NF-κB and c-Fos activation and can also stimulate the formation of more TNF-α (See Figure 2.9, page 49). [181] TNFR requires the recruitment of lipid rafts for TNF-α mediated NF-κB activation, similar to TLR4. [210] By affecting the biophysics of lipid rafts in cell membranes, the LCPUFAs may have affected the function of TNFR. This could have effects on NF-κB and c-Fos activation as well as TNF-α formation. ω-3 LCPUFAs have been shown to decrease the expression of TNF-α in RAW 264.7 murine macrophages, [182] and this may be due to the effect of LCPUFAs on TNFR. It may be suggested that the LCPUFAs in our study exerted their effects through negatively affecting TNFR and this may be the cause of the decrease in c-Fos expression that was recorded. Although not measured, decreases in NF-κB activation and TNF-α formation may have occurred as well. This could further affect osteoclast formation and function.

Rahman *et al*. have shown that exposure to ω-3 LCPUFAs (EPA and DHA) show high levels of incorporation of these fatty acids into the cell in RAW 364.7 murine macrophages. [130] LCPUFAs may therefore have entered the cells and affected key regulators of osteoclast function in this present study. Chan *et al*. have shown that all three PPARs (PPAR-α, PPAR-β/δ and PPAR-γ) are expressed in human CD14+ monocytes. [211] In this study, it was shown that a potent agonist (ciglitizone) for PPAR-γ inhibit osteoclast formation. [211] AA is a potent agonist of PPAR-γ and this may explain why AA showed inhibitory effects in our study. DHA is a less potent agonist of PPAR-γ than AA, [72] and this may explain why AA had more potent effects than DHA in our study. However, PPAR-γ is known to increase osteoclast formation *in vivo* (See Figure 2.8, page 45). Therefore, if the LCPUFAs act as PPAR-γ agonists *in vivo*, they may have detrimental effects on bone health. Recently, Wu *et al*. have shown that a potent PPAR-γ agonist (rosiglitazone) increased osteoclast formation in murine BMMs. [212] As

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Chan *et al* and Wu *et al*. used different types of PPAR-γ agonists, these results may indicate that the type of PPAR-γ agonist plays a role in determining its effects in osteoclasts. Moreover, Chan *et al*. have reported that a ligand for all three PPARs, bezafibrate, inhibited osteoclast formation in human CD14+ monocytes. [211] As stated earlier, ligands for both PPAR-α and PPAR-γ, such as AA and DHA, are known to counteract the effects of PPAR-γ agonists. [73, 166] PPAR-γ is known to up-regulate c-Fos expression (See Figure 2.8, page 45). By counteracting the effects of PPAR-γ, the LCPUFAs tested may cause the decrease in c-Fos expression that was observed in our study. Therefore, it may be suggested that the LCPUFAs tested act as agonists for both PPAR-α and PPAR-γ and therefore cause the inhibitory effects witnessed in this study. AA may be a more potent ligand of PPARs than DHA and this may explain why it showed more potent effects in our study.

In contrast, Yuan *et al*. have reported that AA and EPA enhanced osteoclast formation in murine BMMs; this enhanced formation was decreased in the presence of COX inhibitors. [129] This may indicate that COX metabolites of AA and EPA, such as prostaglandins, may play a role in enhancing osteoclast formation. This same study reported that DHA reduced osteoclast formation but this reduction was reversed by LOX inhibitors. [129] This could strengthen the idea that LOX metabolites of DHA, such as resolvins and protectins, may be involved in the inhibitory effect of LCPUFAs in osteoclasts. AA can be metabolized by LOX to form lipoxins and leukotrienes (See Figure 2.6, page 22) which are known to have bone protective effects. Norris *et al*. have shown that activation of TLR4 may cause murine macrophages supplemented with LCPUFAs to favour LOX metabolism over COX metabolism. [213] As stated earlier, ω-3 LCPUFAs can affect TLR4, [92] and this may increase the formation of LOX metabolites. Therefore it can be hypothesized that the LCPUFAs in this study affected TLR4 thereby favouring their LOX metabolism into lipid mediators that inhibited osteoclast formation and function in the human CD14+ monocytes. The down-regulation of TNF-α induced NF-κB activation by lipoxins may be the method through which they inhibit osteoclast formation and resorption. [187]

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The exact mechanism of action that occurred in the human CD14+ monocytes is unknown. It may be that one or more of the aforementioned mechanisms occur. However, based on previous literature and our findings the following sequence of events may be suggested to have occurred in differentiating osteoclasts and mature osteoclasts (Figure 5.2):

- AA and DHA affect TLR4 or TNFR through incorporation into the cell membrane affecting NF-κB and JNK1 activation.
- LOX metabolites of AA and DHA may affect NF-κB activation. JNK1 and ERK1 pathways in RANK signalling may have been affected by AA and DHA or their metabolites
- AA and DHA may also bind to both PPAR-γ and PPAR-α counteracting the effects of PPAR-γ activation
- Consequently decreased expression of c-Fos which will decrease expression of NFATc1, the master regulator of osteoclasts, will occur
- This will lead to decreases in the gene expression of CTSK, TRAP, MMP9, DC-STAMP and CA2 as witnessed in this study. Possibly due to having more potent effects than DHA, AA also causes decreases in VNR expression
- Decreases in DC-STAMP will be responsible for the decrease in osteoclast formation in differentiating osteoclasts
- Possibly through having a milder or slower effect than AA, genes such as TRAP and CA2 are still expressed in a high amount in mature osteoclasts exposed to DHA
- Decreases in CTSK and MMP9 gene expression lead to decreases in the proteins cathepsin K and MMP-9 required for resorption
- With lower levels of these proteins available, resorption is reduced. Greater decreases in VNR expression, which is required for attachment to bone, may explain why AA showed greater reduction in resorption than DHA
- AA and DHA also affect M-CSF signalling and decrease the expression of the RANK gene. This would lead to decreases in availability of RANK receptors for RANKL, further affecting osteoclast formation and function

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RANKL
TNF-α
M-CSF
AA/DHA incorporation into membrane
Extracellular
RANK
Cytoplasm
TNFR
TRAF 6
TAK1
c-Fms
TLR4
LOX metabolites
NIK
MAPKs
JNK1
p38
ERK1
IKK
AA/DHA or metabolites
NFκB2
? ?
NFκB
c-Fos
c-Jun
PPAR-γ
Nucleus
NFATc1
PPAR-α
Osteoclast differentiation and formation of genes required for osteoclast formation and function: MMP9, CTSK, TRAP, CA2, DC-STAMP, etc.
RANK

Figure 5.2: Possible molecular targets of AA and DHA in human CD14+ monocytes. From previous literature, it is speculated that AA and DHA may affect TLR4 and TNFR through incorporation into the cell membrane. LOX metabolites of AA and DHA may inhibit TNF-α induced NF-κB activation. Our results suggest that JNK and ERK pathways may be affected as well. Activation of both PPAR-γ and PPAR-α may decrease c-Fos expression. M-CSF signalling may also be a molecular target of both LCPUFAs.

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5.4 Recommendations for further studies

Although much is already known about the effects of LCPUFAs on bone health, there are still some areas that need further research. High ω-6 LCPUFA diets have been associated with many side effects. [139] However, in this study we found that AA, an ω-6 LCPUFA, showed potential bone protective effects. Farina *et al*. have shown that men with high AA intake have an 80% lower risk of hip fracture compared to men with low AA intake. [153] This could indicate that the type and/or source of the LCPUFA are crucial in determining its effects. Future studies should focus on investigating the effects of different types and sources of ω-6 LCPUFAs to clarify whether some or all are detrimental to bone health.

It may also be of benefit to explore the individual effects of different doses of ω-3 LCPUFAs (ALA, DHA and EPA) and ω-6 LCPUFAs (LA, AA) separately, to determine normal ranges for optimal bone health of each LCPUFA. These studies could establish whether there are any extra beneficial or detrimental effects of LCPUFAs after the normal ranges have been exceeded. [214] In this study we found that higher concentrations of LCPUFAs (60-80µM) had seemingly harmful effects on differentiating osteoclasts. However, higher levels of LCPUFAs still showed inhibitory effects on mature osteoclast activity without affecting cell numbers. This could indicate that what may be considered optimum LCPUFA dose for mature osteoclasts may not be optimum for pre-osteoclasts. Researchers should keep this is mind when examining normal optimal ranges for bone health.

As we commonly consume more than one type of LCPUFA in our diets, testing different combinations of ω-6 and ω-3 LCPUFA could also help determine the optimal ω-6/ω-3 LCPUFA ratio for bone health. Studies on different combinations may establish whether the type of LCPUFA is important in the ω-6/ω-3 ratio or whether all ω-6 LCPUFAs are detrimental and all ω-3 LCPUFAs are beneficial, *in vivo*.

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More *in vitro* studies should focus on the mechanisms involved in the interaction of LCPUFAs and bone cells in human cell lines. Studies on human osteoclasts, human osteoblasts as well as co-cultures, with both cells grown together, should be the focus of future research. Rahman *et al*. showed that DHA could affect NF-κB signalling in RAW 264.7 macrophages. [130] Evaluating the effect of LCPUFAs on separate pathways (JNK1, ERK1, p-38, NF-κB, etc.) that are triggered by RANK signalling in osteoclasts could further establish the molecular targets of LCPUFAs. The effects of the LCPUFAs on TLR4 and TNFR signalling should be investigated to determine whether the LCPUFAs do target these pathways as well. COX and LOX inhibitors may be used to determine whether the LCPUFAs do elicit their effects through their COX or LOX metabolites. There is also a lack of research on the effects of LCPUFAs on already matured osteoclasts. These studies could further our knowledge on the mechanisms involved between the interaction of LCPUFAs and bone that occur within our own bodies.

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CHAPTER 6

CONCLUSION

In this study, it was shown for the first time that LCPUFAs can affect osteoclast formation and activity in a primary human osteoclast cell line. This study is also the first to examine the effects of LCPUFAs on both differentiating and mature human osteoclasts. The findings suggest that AA and DHA can inhibit osteoclast activity by affecting RANK signalling. Although both LCPUFAs showed an effect on osteoclastogenesis in differentiating osteoclasts, the effect of AA was greater than that of DHA at the same concentration. Smaller and fewer osteoclasts formed however, they appeared normal. It appeared as though the LCPUFAs delayed the formation of osteoclasts in differentiating cells. Delaying the formation of osteoclasts may delay bone loss which could benefit patients with bone diseases with overactive osteoclasts, such as osteoporosis, by giving the osteoblasts more time to replace bone. AA showed a greater effect on resorption in mature osteoclasts than that of DHA. Coupled with its greater effect on protein and gene expression in mature osteoclasts, it appears that AA is a much more potent inhibitor of osteoclast activity than DHA.

As both LCPUFAs affected c-Fos expression, in differentiating and mature osteoclasts, it can be suggested that they target the JNK1 and NF-κB pathway, and possibly the ERK1 pathway (See Figure 5.2). As RANK expression was also affected this could suggest that the LCPUFAs could also affect M-CSF signalling. Although AA and DHA showed similar effects in some of the genes tested, AA showed significantly larger decreases in others (TRAP and CA2). TRAP and MMP-9 protein expression also showed a greater decrease when exposed to AA than DHA. These results may indicate that there could be other pathways that are affected by AA but that are not affected by DHA or vice versa. From these results we can hypothesize that the mechanism through which LCPUFAs prevent bone loss may be through the modulation of osteoclast-

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specific genes leading to a reduction in osteoclast formation and activity. Future studies will evaluate the effect of LCPUFAs on the NF-κB pathway in human osteoclasts. The separate MAPK (JNK1, p-38, ERK1) pathways will be evaluated to determine the exact pathways that are affected by LCPUFAs in human CD14+ monocytes. The effect of the LCPUFAs on other signalling pathways (TLR4, TNFR, etc.) should be evaluated as well. These future studies could also help determine whether LCPUFAs affect all these pathways simultaneously or whether they target specific pathways. This knowledge would further establish the molecular actions of LCPUFAs in osteoclasts. The more potent effect of AA witnessed in this study may also be explained when further pathways are explored.

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APPENDIX A

Ethical Clearance

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Faculty of Health Sciences Research Ethics Committee

13/09/2012

Number	:	**S154/2012**
Title	:	*In vitro* effects of arachidonic acid and docosahexaenoic acid on human CD14+ monocytes
Investigator	:	Abe Edward Kasonga, Department of Physiology, University of Pretoria (SUPERVISORS: Dr M Coetzee / Prof MC Kruger)
Sponsor	:	**MRC**
Study Degree:		**MSc: Human Physiology**

This Student Protocol was reviewed by the Faculty of Health Sciences, Student Research Ethics Committee, University of Pretoria on 13/09/2012 and found to be acceptable. The approval is valid for a period of 3 years.

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Dr MP Mathebula	Deputy CEO: Steve Biko Academic Hospital
Prof A Nienaber	(Female) BA (Hons) (Wits); LLB (Pretoria); LLM (Pretoria); LLD (Pretoria); PhD; Diploma in Datametrics (UNISA)
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Prof T J P Swart	BChD, MSc (Odont), MChD (Oral Path), PGCHE
Prof C W van Staden	**Chairperson** - MBChB; MMed (Psych); MD; FCPsych; FTCL; UPLM; Dept of Psychiatry

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APPENDIX B

Consent Form

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PATIENT INFORMATION LEAFLET AND INFORMED CONSENT

TITLE OF THE STUDY: The influence of fatty acids on bone destruction

Dear Mr ____________________________________ **Date** ___________________

1. INTRODUCTION

We invite you to participate in a research study. This information leaflet is to help you to decide if you would like to participate. Before you agree to take part in this study you should fully understand what is involved. If you have any questions, which are not fully explained in this leaflet, do not hesitate to ask the doctor or investigator. You should not agree to take part unless you are completely happy about all the procedures involved.

2. WHAT IS THE NATURE AND PURPOSE OF THIS STUDY?

The purpose of this study is to determine whether different fatty acids (found in foodstuffs) may improve bone strength. It has been shown that a diet deficient of certain vital fatty acids could lead to bone loss, while dietary supplementation of some fatty acids has been shown to improve bone mass. This laboratory based project will investigate the action of selected fatty acids on bone destruction.

3. WHAT ARE THE PROCEDURES THAT WILL BE FOLLOWED?

You are invited to consider donating 250ml of blood (a cup) that will be collected by an experienced physician from a vein in your arm. We will also measure your blood pressure and determine your BMI. If you have a history of hepatitis or endocrine disease, or are currently on any chronic medication, please inform the phlebotomist as you cannot be considered for donation. Your blood, that contains different types of cells, will be used to prepare specific bone cells in the laboratory. We will then, in the laboratory, investigate the action of selected fatty acids on these newly formed bone cells. All blood samples will be numbered and anonymity is guaranteed and results will not be back-traceable to any specific patient. This study is primarily laboratory based and biochemical in nature.

4. WHAT ARE THE RISKS INVOLVED IN THIS RESEARCH STUDY?

The only possible risk and discomfort involved is the taking of blood from a vein and the measurement of vitals. However, please refrain from donating blood for at least a month after this donation. You can contact Dr Craig Grobbelaar (Telephone number: 012 319 2136) if you have any queries about donating blood.

5. WHAT ARE THE POSSIBLE BENEFITS OF THE STUDY?

Although you will not benefit directly from the study, information gained from this study could help to develop new treatments for osteoporosis.

6. WHAT IS THE DURATION OF THIS STUDY?

If you decide to take part in this study, you will be one of approximately 20 patients. The blood collected in this way will enable the investigators to do laboratory based and biochemical research. Results may only become available after a few years.

7. WHAT ARE YOUR RIGHTS AS A PARTICIPANT IN THIS RESEARCH STUDY?

Your participation in this research study is entirely voluntary and you can refuse to participate without stating any reason.

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8. HAS THE RESEARCH STUDY RECEIVED ETHICAL APPROVAL?

This study has received written approval from the Research Ethics Committee of the Faculty of Health Sciences, University of Pretoria. A copy of this letter may be obtained from the investigator should you wish to review it. You can contact the Research Ethics Committee of the Faculty of Health Sciences (Telephone number: 012 354 1330 or 012 354 1677) if you have any enquiries about the ethical nature of the project.

9. INFORMATION AND CONTACT PERSON

Should you require consulting further on the purpose and the procedures of this study you can contact Dr M Coetzee (Telephone number: 012 319 2445, Cell phone: 076 835 5677).

10. COMPENSATION

You will receive a remuneration of R50 for out-of-pocket expenses immediately after the blood has been collected.

11. CONFIDENTIALITY

All information obtained during the course of this research study is strictly confidential. Data that may be reported in scientific journals will not include any information that might identify you in this research study. All collected materials will be destroyed after completion of the study.

Any information uncovered regarding your test results or state of health as a result of your participation in this research study will be held in strict confidence. Information in a form where you can be identified will not be disclosed to any third party without your written permission.

12. CONSENT TO PARTICIPATE

I hereby confirm that I have been informed by_____________________________, regarding the nature, conduct, benefits and risks of the research study. I have also received, read and understood the above written information (Patient Information Leaflet and Informed Consent) regarding the research study.

I am aware that the results of the research study, including personal details regarding my sex, age, date of birth, initials and diagnosis will be anonymously processed into a research report.

I may, at any stage, without prejudice, withdraw my consent and participation in the research study. I have had sufficient opportunity to ask questions and (of my own free will) declare myself prepared to participate in the research study.

Patient's Name ___________________________________

(Please print)

Patient's Signature ____________________________________ Date ________________

Doctor’s Name ____________________________________

Doctor’s Signature ____________________________________ Date ________________

I, _______________________________ herewith confirm that the above patient has been informed fully about the nature, conduct and risks of the above research study.

Witness's Name _______________________________________

Witness's Signature _____________________________________ Date _________________

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