In vitro effects of palmitoleic acid on osteoblast differentiation in MG-63 osteosarcoma cells and human adipose-derived stromal cells

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In vitro effects of palmitoleic acid on osteoblast differentiation in MG-63 osteosarcoma cells and human adipose-derived stromal cells

by

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Dedication

This dissertation is dedicated to my late mother Keyden, my father Andrew, my twin sister Staci-Leigh, my brother Wesley, Rogan Bruce, and my family. Thank you for your continuous love and encouragement throughout my studies. I could never have achieved this without your support.

Declaration

I, Kayla Howard, declare that this dissertation, which I hereby submit for MSc in Human Physiology at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signature:  _____ Date: 19 November 2019

Ethics statement

I, Kayla Howard, declare that I have obtained ethical clearance for the research described in this work from the Faculty of Health Sciences Ethics Committee (386/2018). I declare that I have observed the ethical standards required in terms of the University of Pretoria's Code of ethics for researchers and the policy guidelines for responsible research.

Signature: 

Date: 19 November 2019

Summary

Bone is an important organ influenced by mechanical load, hormones, nutrition and disease. During bone remodelling, osteoclasts resorb bone and osteoblasts form new bone. Osteoblasts are derived from mesenchymal stem cells (MSCs) such as adipose-derived stromal cells (ASCs). The mitogen-activated protein kinase (MAPK) pathway has been shown to interfere with osteoblast differentiation from an early stage. Runt related transcription factor 2 (RUNX2) exerts an effect downstream from p38 MAPK. RUNX2 phosphorylation by p38 MAPK may increase osteoblast differentiation markers such as alkaline phosphatase (ALP), osteoprotegerin (OPG) and receptor activator of nuclear factor kB ligand (RANKL). Palmitoleic acid (PLA), an omega-7 monounsaturated fatty acid (MUFA), promotes anti-osteoclastogenic effects, however, the effects of PLA on osteoblasts has not been reported.

Osteoporosis is a condition which has debilitating effects in the elderly. Unsaturated fatty acids (UFA) have been studied for their beneficial effects on human health for a number of years. Polyunsaturated fatty acids (PUFA) have been studied as a potential therapeutic agent to prevent and assist in managing the condition. Few studies have been conducted on the effects of MUFA on bone therefore this study aimed to investigate the effects of PLA on osteoblast differentiation using ASCs and MG-63 osteosarcoma cells as an osteoblast model.

ASCs and MG-63 osteosarcoma cell lines were exposed to PLA (20-100 μM) in osteogenic media (OM). The effects of PLA on cell viability was evaluated on undifferentiated cells. Thereafter, cells were exposed to PLA for 7, 14 or 21 days. Subsequently ALP activity, calcium mineralisation, gene expression, protein expression and adipogenesis were assessed.

In this study, PLA had no significant effects on cell viability in undifferentiated cells. Furthermore, PLA had no significant effects on ALP activity, calcium mineralisation or phosphorylated extracellular signal-regulated kinase (pERK)/extracellular signal-regulated kinase (ERK) expression in differentiating cells, however, ALP activity increased at 7 day in ASCs and 21 days in MG-63 cells. Alizarin Red S staining increased at 21 days in both cell lines with a significant increase in the ASCs, however, calcium nodules were not visible. In the ASCs, PLA significantly increased the gene

expression of ALP at 7 and 14 days compared to control ($p<0.01$ and $p<0.05$) while RANKL was significantly decreased at 7 days compared to the control ($p<0.05$). In the MG-63 cells, RUNX2 and OCN were significantly reduced at 7 days compared to control ($p<0.05$) and ALP, RUNX2, Osx and RANKL were significantly reduced at 14 days compared to control ($p<0.001$ and $p<0.05$). In the ASCs, lipid accumulation was not present after 21 days while in MG-63 cells, there was a significant increase in lipid accumulation at a high concentration of PLA after 21 days compared to control ($p<0.05$).

This is the first study to explore the effects of PLA on osteoblast formation using ASCs and MG-63 osteosarcoma cells. Results suggest that PLA exerted changes in the ASCs and MG-63 cells during osteoblast differentiation, however, these changes were not significant. To conclude, PLA showed some significant effects on osteoblast-specific gene expression, however, most of the osteoblast-specific gene expression was downregulated, particularly in the MG-63 cells, after PLA treatment.

Keywords: Bone remodelling, osteoblasts, adipose-derived stromal cells, MG-63 osteosarcoma cells, monounsaturated fatty acids, palmitoleic acid, alkaline phosphatase, runt-related transcription factor, osteoprotegerin, mitogen-activated protein kinase.

Research outputs

Conference outputs

Poster presentation: Faculty of Health Sciences Research day, University of Pretoria, August 2018.
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List of abbreviations

AA	Arachidonic acid
ALA	α-linoleic acid
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
APC	Adenomatosis polyposis coli
ASCs	Adipose-derived stromal cells
BCA	Bicinchoninic acid
BDP	4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s indacene
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
BMP	Bone morphogenic proteins
BMPR	Bone morphogenic protein receptor
BMU	Basic multicellular unit
BSA	Bovine serum albumin
BSP	Bone sialoprotein
CD	Cluster of differentiation
CDK1	Cyclin dependent kinase 1
cDNA	Complementary deoxyribonucleic acid
CEBPα	CCAAT/enhancer binding protein alpha
COL1	Collagen type 1
Co-Smads	Common Smads
CXCR4	C-X-C chemokine receptor type 4
ddH_2O	Double-distilled water
DGLA	Dihomo-γ-linoleic acid
DHA	Docosahexaenoic acid
DMEM	Dulbecco’s Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPA	Docosapentaenoic acid

DXA	Dual X-ray absorptiometry
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EPA	Eicosapentaenoic acid
EPP	Padina pavonica extract
ERK	Extracellular signal-regulated kinase
EZH2	Enhancer of zeste homolog 2
FA	Fatty acids
FBS	Foetal bovine serum
FDA	Food and Drug Administration
FFA	Free fatty acids
FGF	Fibroblast growth factors
Fzd	Frizzled
GAPDH	Glyceraldehyde-3-phosphate
GH	Growth hormone
GHR	Growth hormone receptor
GLA	Gamma-linoleic acid
GPCR	G-protein coupled receptors
GSK3β	Glycogen synthase kinase 3β
HDL	High-density lipoprotein
Hh	Hedgehog
HRP	Horseradish peroxidase
IGF	Insulin-like growth factors
Ihh	Indian hedgehog
IKKβ	Nuclear factor kappa-B kinase subunit beta
IL	Interleukin
I-Smads	Inhibitory Smads
IκB	Inhibitor of nuclear factor κB
JAK/STAT	Janus kinase/signal transducers and activators of transcription
JNK	Jun N-terminal kinases
LA	Linoleic acid
LCPUFA	Long-chain polyunsaturated fatty acids
LDL	Low-density lipoprotein
LRP	Low-density lipoprotein receptor-related protein

MA	Myristic acid
MAPK	Mitogen-activated protein kinase
M-CSF	Macrophage colony stimulating factor
MiRNA	Micro-ribonucleic acids
mRNA	Messenger ribonucleic acid
MSCs	Mesenchymal stem cells
MUFA	Monounsaturated fatty acids
NF-κB	Nuclear factor kappa-B
NLK	Nemo-like kinase
NR	9-Diethylamino-2-hydroxy-5H-benzo (α) phenoxazine-5-one
OA	Oleic acid
OCN	Osteocalcin
Oct-4	Octamer-binding transcription factor 4
OM	Osteogenic media
ON	Osteonectin
OPG	Osteoprotegerin
OPN	Osteopontin
Osx	Osterix
OVX	Ovariectomised
PA	Palmitic acid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factors
pERK	Phosphorylated ERK
PGE_2	Prostaglandin E_2
PGI_2	Prostacyclin
pJNK	Phosphorylated JNK
PLA	Palmitoleic acid
PMSF	Phenylmethane sulfonyl fluoride
pp38	Phosphorylated p38
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PPRE	Peroxisome proliferator-responsive element
PTH	Parathyroid hormone
PTHrP	Parathyroid related protein

PUFA	Polyunsaturated fatty acids
qPCR	Quantitative polymerase chain reaction
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
RNA	Ribonucleic acids
ROS	Reactive oxygen species
R-Smads	Regulatory Smads
RUNX2	Runt related transcription factor 2
SA	Stearic acid
SCD-16	Delta-9-desaturase/stearoyl-CoA desaturase
SDF-1	Stromal cell-derived factor 1
SERMs	Selective oestrogen receptor modulators
SETD1	SET domain containing 1
SFA	Saturated fatty acids
Shh	Sonic hedgehog
Smad1	Smad family member 1
SOST	Sclerostin
SOX-2	Sex determining region Y-box 2
SSEA-4	Stage-specific embryonic antigen-4
TAB-1/2	TAK-1 binding protein 1/2
TAK-1	Mitogen-activated protein kinase kinase kinase 7/ transforming growth factor beta-activated kinase 1
TAZ	Tafazzin
TBS	Tris-buffered saline
TBS-T	Tris-buffered saline with tween
TCF/LRF	T-cell factor/lymphoid enhancer factor
TGF-β	Transforming growth factor β
TNF-α	Tumour necrosis factor α
UFA	Unsaturated fatty acids
v/v	Volume per volume
VC	Vehicle control
VC+OM	Vehicle control in the presence of osteogenic factors
w/v	Weight per volume
Wnt	Wingless-related integration sit

Chapter 1

Introduction

1.1 Rationale for research

Bone is a dynamic organ that is continuously being resorbed and reformed in a process called bone remodelling. This occurs as a response to mechanical loading, hormonal changes or damage.[1] Imbalances between bone resorption and bone formation could lead to a skeletal condition known as osteoporosis.[2] This disorder ultimately leads to bone fragility which may be due to an increase in bone resorption or a decline in bone formation.[2] The process of bone remodelling is primarily carried out by bone resorbing osteoclasts and bone forming osteoblasts.[2] In 2015, 20 million individuals over the age of 50 were estimated to be living with osteoporosis in 6 European countries (France, Germany, Italy, Spain, Sweden and United Kingdom).[3] Finding new ways to treat, alleviate or slow down the progression of osteoporosis and other bone weakening disorders are important.

Mesenchymal stem cells (MSCs) are pluripotent cells found in bone marrow, the periosteum, the umbilical cord, vessel walls, liver, muscle and fat tissues and are able to differentiate into multiple lineages, one of which is osteoblasts.[4, 5] Adipose-derived stromal cells (ASCs) is a population of stem cells found primarily in fat tissue in which osteoblastogenesis can be induced using osteogenic medium (OM) which contains β-glycerophosphate, ascorbic acid and dexamethasone.[6]

Dietary fat contains a combination of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) which are categorised according to the number of double bonds in their carbon chain.[7] *In vitro*, various studies have been conducted on the effects of unsaturated fatty acids (UFA) on osteoporosis, cancer, cardiovascular disease and bone health.[8, 9] The beneficial effects of PUFA, especially long-chain polyunsaturated fatty acids (LCPUFA), on bone health has shown promising results.[10] Human and animal studies have been conducted to demonstrate the effects of a low omega-6/omega-3 fatty acids (FA) ratio

in maintaining bone mineral density (BMD).[8] MUFA are considered non-essential FA as they can be formed endogenously as well as present in vegetable oils such as olive oil, sunflower oil and rapeseed oil.[7] In a study by Gillingham *et al*., a MUFA rich diet, such as the Mediterranean diet, contributed to a reduction in coronary heart disease and chronic diseases.[11] Previously, palmitoleic acid (PLA), a MUFA found in macadamia oil, has been shown to inhibit osteoclast formation, however, there are currently no published studies on the effects of PLA on human osteoblast cell lines.[12]

1.2 Purpose of research

The aim of this study was to investigate the effects of PLA on osteoblast differentiation in pre-osteoblastic ASCs and MG-63 osteosarcoma cells and to elucidate a possible mechanism of action of this FA in osteoblast differentiation.

1.3 Method of investigation

The study was conducted *in vitro* on ASCs isolated from processed lipoaspirates and pre-osteoblastic MG-63 osteosarcoma cells. The cells were exposed to OM and PLA for 7, 14 and 21 days to test their effects on osteoblast formation. The effects of PLA on alkaline phosphatase (ALP) activity, mineralisation, gene and protein expression and adipogenesis were investigated.

1.4 Objectives

1. **Cell viability**

   To test the effects of PLA on cell viability in undifferentiated ASCs and MG-63 osteosarcoma cells by using a resazurin colorimetric assay.

2. **Alkaline phosphatase activity**

   To examine the effects of PLA on ALP activity in differentiated osteoblasts derived from ASCs and MG-63 osteosarcoma cells as an early marker for osteoblastogenesis, by colorimetric assay.

3. Mineralisation

To study the effects of PLA on mineralisation in differentiated osteoblasts derived from ASCs and MG-63 osteosarcoma cells, using Alizarin Red S staining.

4. Gene expression

To assess the effects of PLA on gene marker expression in differentiating ASCs and MG-63 osteosarcoma cells, using quantitative polymerase chain reaction (qPCR).

5. Protein expression

To investigate the effects of PLA on protein marker expression in differentiating osteoblasts derived from ASCs and MG-63 osteosarcoma cells, using Western blotting.

6. Adipogenesis

To measure the effects of PLA on adipogenesis in differentiated osteoblasts derived from ASCs and MG-63 osteosarcoma cells, using Oil Red O staining.

Chapter 2

Literature review

This section will begin with a short description of bone, its structure and function; the types of cells which make up bone; and the bone remodelling cycle along with the consequences of an imbalanced bone remodelling cycle resulting in osteoporosis. Pathways involved in the differentiation of osteoblasts as well as adipocytes will be discussed with emphasis on the mitogen-activated protein kinase (MAPK) pathways in osteoblastogenesis. Fatty acid structure and classification will lastly be discussed, focusing on PUFA, MUFA and PLA in particular, and their effects which have been studied in both animal and human bone health. This will allow a better understanding of our current knowledge and what the study hopes to achieve.

2.1 Bone

The human skeleton consists of four main categories of bone, namely long bones such as fibulae, tibiae, ulnae and radii; short bones such as patella, tarsal and carpel bones; flat bones such as the mandible, ribs and sternum; and irregular bones such as vertebrae, coccyx and sacrum.[13] Bone provides the body with structural support, protects organs, contributes to mineral homeostasis and acid-base balance and houses the bone marrow where blood cells are produced.[13, 14] Bone comprises of three basic components.[15] The first component is mineralized type I collagen (COL1) fibrils which contain the protein collagen. It is also found in other soft tissues including tendons and skin.[16] The second component is hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) and the third component is water.[16-18] Collagen fibrils merge with adjacent fibrils and each fibril has an average diameter of 80-100 nm.[19, 20] Fibrils cluster together to form a larger fiber hence they never occur alone.[15] Hydroxyapatite crystals are found to be plate-shaped with a length and width of approximately 50 x 25 nm.[21-23] Water is found between fibrils as well as within fibrils. It is also present in any gaps between fibers.[17] The relative proportions between water, COL1 and minerals vary between different types of bone.[24] A raise in mineral content generally displays a decline in water content while the amount of COL1 typically remains the same.[15]

In humans, bone is categorised into compact (cortical) and spongy/trabecular (cancellous) bone. Compact bone is dense and surrounds the bone marrow space.[13] It is arranged into systematic concentric lamellae.[25] Haversian systems are osteons which form a network within compact bone.[13] Trabecular bone, on the other hand, is structured into an irregular honeycomb network of rods and plates (Figure 2.1).[13, 26] Trabecular bone is more metabolically active than compact bone therefore it undergoes bone remodelling more often.[13, 27] Different skeletal sites have various cortical to trabecular ratios therefore the proportion of cortical and trabecular bone significantly affects bone strength.[13] Osteons have a semilunar structure and are composed of concentric lamellae.[13] Interstitial lamellae are found in the space between osteons and are composed of secondary osteons which have been partially resorbed and not removed during bone remodelling.[28]

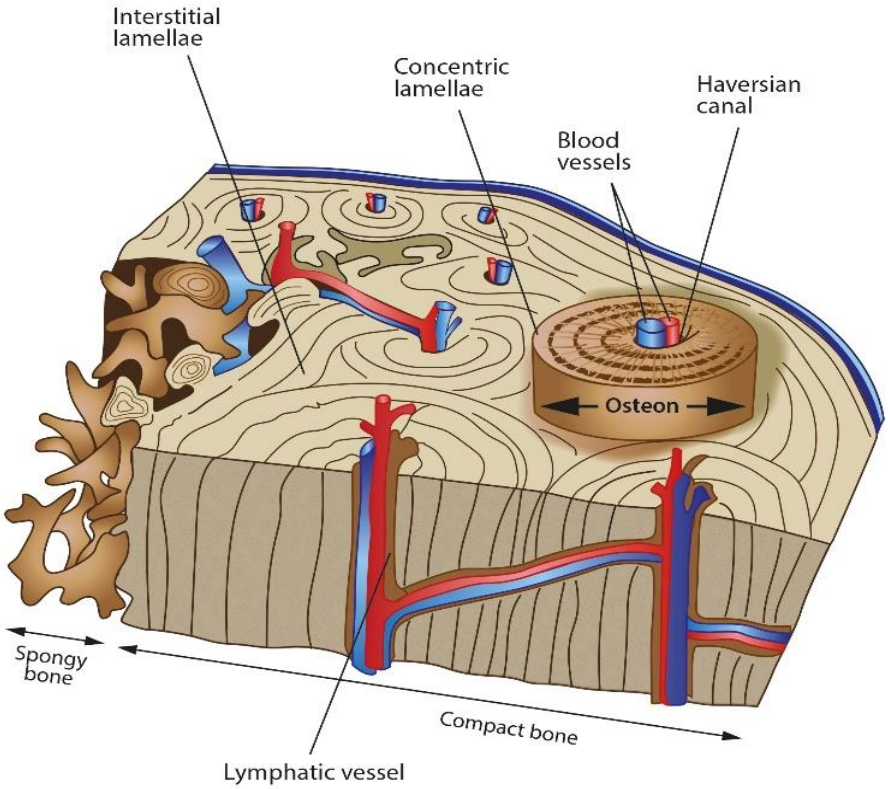

Figure 2.1. The organisation of compact and spongy bone.

Cortical (compact) bone surrounds the bone marrow space and contains osteons which are made up of concentric lamellae surrounding a Haversian canal. Haversian canal's contain lymphatic vessels and blood vessels to supply bone with nutrients and to get rid of waste materials. Trabecular (spongy) bone is made up from a honeycomb network of rods and plates. Image created with information from Gunnarsson *et al*. (2013).[26]

In a long bone, the ends consist of dense compact bone on the outer shell with a spongy trabecular interior.[13, 27] In a long bone, the diaphysis is composed of a hollow shaft and is mainly composed of cortical bone (Figure 2.2).[29] Located below the epiphyseal growth plate is the cone-shaped metaphysis while the round epiphysis is located above the epiphyseal growth plate. These are primarily composed of a meshwork of trabecular bone enclosed by a thin layer of cortical bone.[13] The periosteum surrounds the outer surface of long bones and is made up of a fibrous connective tissue sheath. It contains blood vessels, nerve fibres and cells involved in bone remodelling.[13] The periosteum attaches to the external surface of bone by Sharpey's fibres while the endosteum, a membrane which covers the internal surface of cortical and trabecular bone, lines the medullary cavity and encloses the Volkman's canals (blood vessel canals).[13, 29] The endosteum contains blood vessels and cells involved in bone remodelling.[13, 29] Flat bones have dense outer layers of compact bone and a thin trabecular structure within.[27] Irregular bones such as vertebrae resembles a long bone in the anterior vertebral body, consisting of spongy bone surrounded by a thin layer of cortical bone. The posterior vertebral arch consists of laminae, which are flattened bone with a dense cortex and less dense spongy bone; and pedicles which have dense cortices and less dense spongy bone.[30]

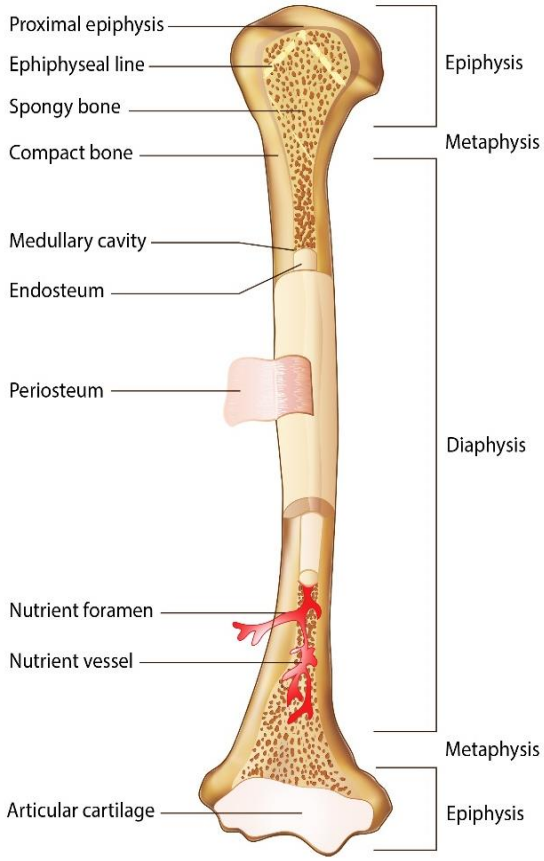

Figure 2.2. The structure of a long bone.

A long bone which shows the compact bone surrounding the spongy bone. The periosteum is attached to the external bone surface while the endosteum is attached to the internal surface of the bone and encompasses blood vessels important for nutrient delivery. Image created with information from Markings *et al*. (1995).[29]

2.1.2 Bone remodelling

Bone remodelling is a continuous process whereby bone is resorbed and then reformed again.[1, 31] This process occurs in order to strengthen and repair bone which has been weakened due to mechanical loading or injury. It similarly assists with calcium homeostasis in the body. Bone remodelling takes place at trabecular and cortical bone sites and is conducted by osteoclasts, osteoblasts and osteocytes.[1] A basic multicellular unit (BMU) mainly consists of osteoclasts and osteoblasts which are present during a bone remodelling cycle (Figure 2.3).[32] Activation, bone resorption, reversal, bone formation and quiescence are five phases which characterise the bone remodelling cycle.[32] The *activation phase* is initiated by either mechanical loading, structural damage or changes in paracrine factors. Structural damage or increased mechanical loading causes a decrease in transforming growth factor β (TGF-β). Bone

lining cells are also activated which results in the recruitment of osteoclast precursors.[32] Lining cells are terminally differentiated osteoblasts which remain on the bone surface and they play a role in recruiting osteoclast precursors.[1, 32, 33] Osteoclast precursors express receptor activator of nuclear factor kappa-B (RANK) which either binds to RANK ligand (RANKL) expressed by osteoblasts to activate osteoclast formation, or to osteoprotegerin (OPG) which inhibits osteoclast formation.[34]

Osteoclasts are multinucleated cells derived from hematopoietic stem cells which resorb bone by forming a sealed micro-environment where acid and proteases are secreted to degrade and demineralize the bone matrix during the *bone resorption* phase.[32] During the *reversal phase*, undigested bone matrix fragments are removed from the bone surface.[32] The degraded matrix causes local paracrine signals to be released which subsequently results in the recruitment of osteoblasts to initiate the *bone formation* phase.[32] New bone matrix is secreted and mineralized by mature osteoblasts to cover the resorption cavity.[32] Once bone formation has been completed, the bone surface returns to a state of *quiescence* where bone lining cells remain on the surface.[32] Bone remodelling is maintained by means of balancing bone resorption and bone formation.[1, 35] An imbalance of these processes can lead to bone disorders such as osteopetrosis, osteoporosis and Paget's disease.[1] Osteopetrosis is an uncommon genetic disorder characterised by defective osteoclast function resulting in an increase in bone mass. This leads to the accumulation of old bone and spontaneous fractures.[36] Paget's disease is a common skeletal disorder caused by accelerated and disorganised bone remodelling in either one or more skeletal sites.[37]

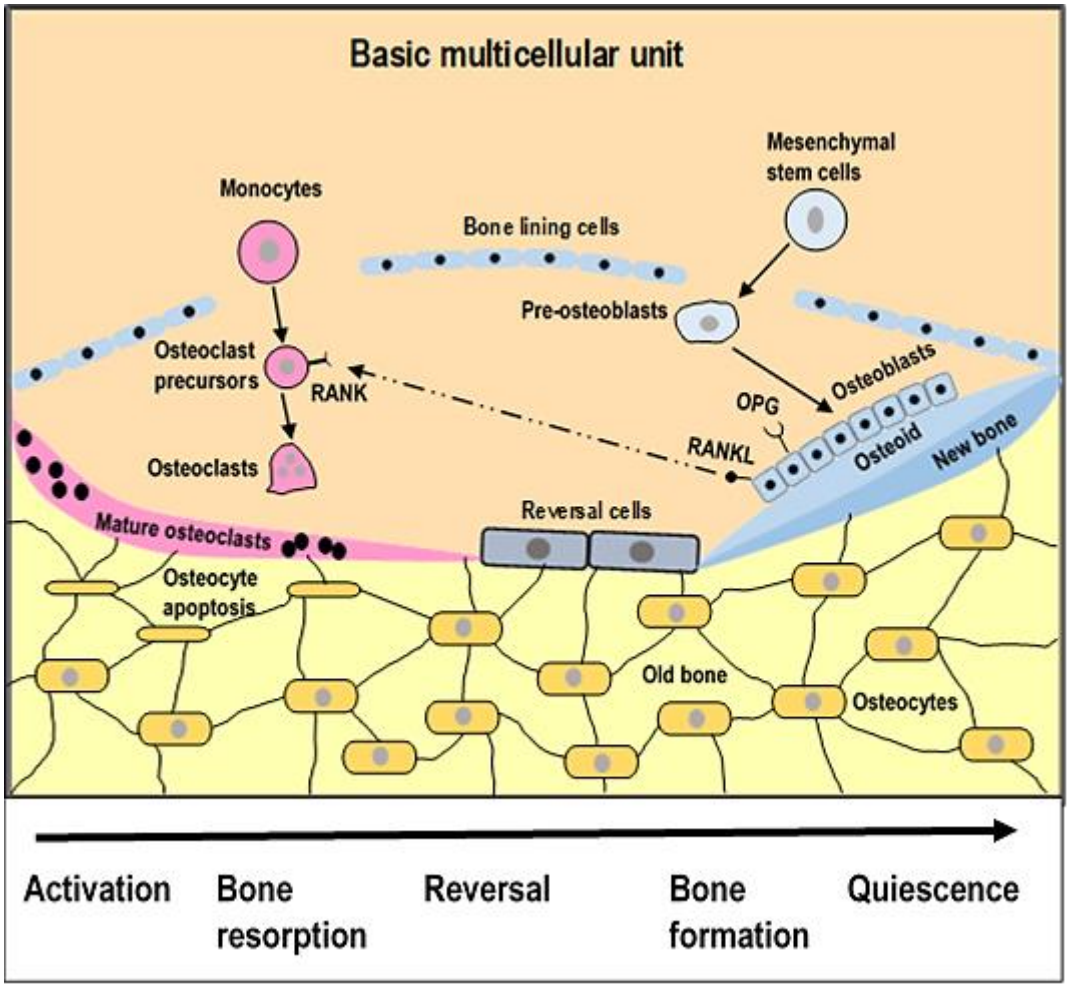

Figure 2.3. Basic multicellular unit during a bone remodelling cycle

There are five phases in a bone remodelling cycle, namely activation, bone resorption, reversal, bone formation and quiescence. Osteocytes, found embedded within the mineralized matrix, sense microdamage and initiates the remodelling cycle. Osteoclast precursors express RANK which binds to RANKL expressed by osteoblasts. Osteoclasts differentiated from hematopoietic stem cells resorb bone. Osteoblasts which differentiate from MSCs, express OPG and mineralize new bone matrix. Image created with information from Nicholls *et al*. (2012).[32]

2.1.3 Bone cells

Osteoclasts

Osteoclast precursors express RANK which binds to RANKL to stimulate osteoclast formation.[32] OPG exerts its osteoclast inhibitory effect by blocking the RANK and RANKL interaction (Figure 2.3).[32, 34, 38, 39] During bone resorption, osteoclasts release factors, that become trapped within the bone matrix, which could help stimulate bone formation by osteoblasts.[1] These factors include insulin-like growth factors I and II (IGF)[40], fibroblast growth factors (FGF)[41], TGF-β1 and 2[42], bone morphogenic proteins

(BMP) 2,3,4,6, and 7[43-45], and platelet-derived growth factors (PDGF)[46]. Once bone resorption is complete, osteoclasts undergo apoptosis and are removed from the bone surface.[1] The concentration of these factors released by osteoclasts could determine the quantity of bone which could be resorbed.[1] An imbalance between osteoclastic bone resorption and osteoblastic bone formation could lead to osteoporosis which is characterised by low bone density or bone mass leading to weaker bone prone to fractures.[47]

Osteoblasts

Osteoblasts are cells derived from MSCs which produce an unmineralised extracellular matrix (ECM), consisting of mainly COL1, known as osteoid which becomes mineralized into new bone matrix by forming hydroxyapetite.[1, 13, 31, 47] The secretion of osteoid slows down as the bone remodelling cycle finishes.[1] Osteoblasts become trapped within the bone matrix after bone formation and differentiate into osteocytes or bone lining cells.[1, 47, 48]. Osteoblasts secrete OPG, which acts as an osteoclast inhibitor as it is a decoy receptor for RANKL.[13] A high OPG/RANKL ratio impedes bone resorption and osteoclast activity while a low OPG/RANKL ratio stimulates bone resorption and osteoclast activity.[9]

Osteoblasts similarly secrete non-collagenous proteins such as osteocalcin (OCN), osteopontin (OPN) and osteonectin (ON).[1] OCN plays a role in regulating glucose homeostasis and binds to the bone matrix where it undergoes decarboxylation and becomes activated during resorption. Once activated, OCN enhances insulin release from beta cells in the pancreas, which in turn enhances OCN production once again.[49] Maximal expression of OCN is associated with matrix mineral deposition at a terminal stage of osteoblast differentiation.[49] OPN is a protein which has been deposited in the matrix in a previous bone formation cycle by osteoblasts and binds to hydroxyapatite. The accumulation of OPN on the bone surface attracts osteoclasts and their precursors. OPN assists in forming a close-fitting attachment between osteoclasts and the bone surface to allow bone resorption to take place.[50] ON is a glycoprotein which supports bone remodelling and plays a role in the maintenance of bone mass.[51] *In vitro* studies have shown that ON binds to collagen and hydroxyapatite.[52, 53]

Osteoblast differentiation may be affected by ephrins, parathyroid hormone related protein (PTHrP) and sclerostin (SOST) amongst others.[1] EphrinB2 is a transmembrane protein produced by mature osteoblasts and assists in the communication with surrounding osteoblasts.[54] EphrinB2, also found on osteoclast precursors, binds to the EPHB4 receptor in osteoblast precursors to stimulate the differentiation of osteoblasts (Figure 2.4).[54] As a result, disrupting EphrinB2 and EPHB4 binding impairs bone mineralisation.[1, 54] Osteoblast proliferation and differentiation plays a crucial role in skeletal homeostasis and fracture healing.[47]

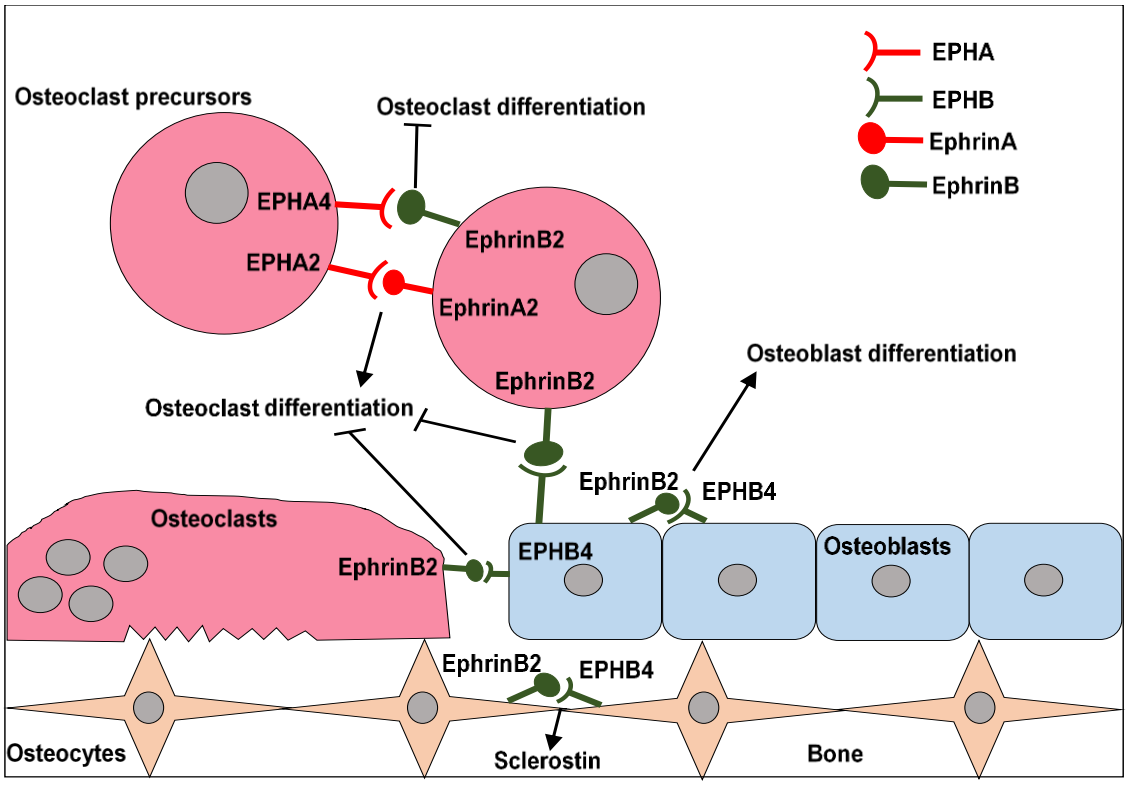

Figure 2.4. The interaction between EphrinB2 and EPHB4.

Osteoclast formation is inhibited by the interactions of EPHA4 and EphrinB2 which stimulates osteoblast differentiation. EPHA2 and EphrinA2 inhibits osteoblast formation and stimulates osteoclast differentiation thus it has the opposite effect. Osteoblast differentiation is also enhanced by EphrinB2/EPHB4 interactions within osteoblast lineages. Osteocytes express EphrinB2 and EPHB4 and interference with this interaction inhibits the expression of SOST. Image created from information by Sims *et al*.[54]

Osteocytes

Osteocytes are considered to be mechanosensors as they appear to respond to mechanical stress placed on bone.[13, 48] Osteocytes are connected to other osteocytes

to sense microdamage within the bone matrix and play a role in the bone remodelling process.[1] Under basal conditions, TGF-β and SOST are secreted by osteocytes which inhibits osteoclast differentiation in addition to wingless-related integration site (Wnt)-activated bone formation by osteoblasts.[32] Osteocytes may also express macrophage colony-stimulating factor (M-CSF) and RANKL which are essential for the formation of osteoclasts.[32] Osteocytes release SOST which inhibits bone formation. SOST interferes with the BMP and Wnt signalling pathways to induce osteoblast apoptosis, impair bone mineralisation and inhibit the proliferation of osteoblasts.[1]

2.2 Osteoporosis

Osteoporosis is a condition marked by general bone fragility attributed to a reduction in bone quality and quantity where bone strength is considerably reduced causing fractures without significant trauma.[2, 55] Skeletal integrity and peak bone mass is influenced by genetic, hormonal, physical and nutritional factors.[55] In the United States, 1.5 million fractures occur per year as a consequence of osteoporosis with a majority occurring in postmenopausal women.[56] Recent statistics for South Africa regarding osteoporosis is not available however, by 2050, an estimated 28% (13.6 million) of South Africans will be over 50 years of age while 6% (4 million) of the population will be over 70 years of age with the total population remaining around 50 million.[57] Osteoporosis and fractures related to osteoporosis is a concern for public health as it results in massive health care costs as treatments are costly.[58]

2.2.1 Pathogenesis

Bone mass is acquired mainly between the ages of 12-18 years of age.[2] Recent studies have shown that BMD may begin to decrease from as early as 30 years of age in both men and women, however, women experience accelerated BMD loss during menopause and thereafter.[2, 58] The mechanism of this pathogenic condition can be attributed to either failing to achieve a high peak bone mass and bone strength during puberty and growth; an increase in bone resorption by osteoclasts; or insufficient bone formation by osteoblasts causing a decrease in bone mass and strength.[59]

There are two subtypes of primary osteoporosis namely osteoporosis type I and type II, and one type of secondary osteoporosis.[60] Type I osteoporosis is generally due to hormone loss in postmenopausal women triggered by oestrogen deficiency, and is a

result of enhanced osteoclast activity mainly affecting trabecular bone sites.[60] Type II osteoporosis results from a decline in osteoblast function commonly brought on by the aging of cortical and trabecular bones, leading to a loss of bone mass.[47, 60] Secondary osteoporosis, in contrast, is typically brought on by other conditions such as osteogenesis imperfecta, type 2 diabetes mellitus, end-stage renal disease, or lifestyle risks which include insufficient vitamin D consumption, immobilisation, low calcium intake, smoking and alcohol abuse.[60] Glucocorticoid therapy, used for the treatment of chronic diseases, exerts a detrimental effect on bone by decreasing osteoblast differentiation, impairing osteoblast function, reducing the lifespan of osteoblasts and increasing the duration of osteoclast activity and osteoclast formation.[61]

Osteoporosis can be diagnosed by determining BMD using dual X-ray absorptiometry (DXA) which measures the grams of mineral calcium present per square centimeter of a bone.[60] This is typically performed on the hip or spine to predict fracture risks in the future.[60] A T-score is then calculated based on the difference between the patient's BMD and the mean BMD of young females between the ages of 20-29 years of age. This difference is subsequently divided by the standard deviation of a reference population. If the T-score of the BMD is 2.5, or more standard deviations below the mean for young females, the patient is diagnosed with osteoporosis.[60] Osteopenia is present if the patient has a T-score of between 1 and 2.5 standard deviations below the mean of a healthy population of females.[60]

Age-related osteoporosis is typically caused by the senescence of bone cells and changes in hormone levels.[61] It is commonly marked by a decrease in cortical thickness, frequently seen in trabecular bone.[61] Bone deterioration may be due to several factors including age-related osteoblast proliferation impairment; decreased osteoblast lifespan; reduced osteoblast differentiation and function; a diminished response to growth factors and hormones; the differentiation of MSCs into adipocytes rather than osteoblasts; and osteoblast senescence.[61] This contributes to increased bone marrow adiposity observed in the elderly.[61]

In postmenopausal women, oestrogen levels decrease rapidly which results in an increase in both bone resorption and bone formation with resorption increasing more than formation.[58] This is due to the BMU becoming increasingly activated at additional sites on the bone surface and the resorption time by osteoclasts being extended while

bone formation time by osteoblasts are shortened.[58] This has a negative effect on calcium homeostasis in the body as an efflux of calcium into the extracellular fluid from resorbed bone will cause compensatory mechanisms to be stimulated therefore decreasing the uptake of renal calcium, decreasing intestinal calcium absorption and reducing parathyroid hormone secretion (PTH).[58] Furthermore, oestrogen is known to suppress RANKL expression and increase OPG expression as a bone protective mechanism to limit osteoclast development and activity. During menopause, this reduction in oestrogen reduces the OPG/RANKL ratio which results in increased osteoclast formation and excessive resorption.[58] Oestrogen also promotes the differentiation of MSCs into osteoblasts as well as reduces osteoblast apoptosis.[58]

2.2.2 Treatment

Apart from pharmacological treatment, non-pharmacological options are available to diminish the progression of osteoporosis and assist in managing the condition.[56] Evaluating lifestyle risk factors such as smoking or excessive alcohol consumption may be beneficial in osteoporosis treatment and management.[56] Exercise in addition to adequate calcium and vitamin D intake may similarly assist in reducing the fracture risk of patients with osteoporosis.[56] There are several pharmacologic therapies used in the treatment of osteoporosis, however, these therapies may cause adverse side effects.[56]

Oestrogen and selective oestrogen receptor modulators (SERMs) are hormone and hormone analogues which bind to the oestrogen hormone receptor to cause a response.[58] These hormone therapies given to postmenopausal women were shown to prevent bone loss through anti-resorptive effects, however, it may have non-skeletal adverse effects such as increasing the chance of developing breast cancer, or causing thrombotic, cerebrovascular and coronary events.[56, 60]

Bisphosphonates are inorganic pyrophosphate analogues which have a high binding affinity to hydroxyapatite causing osteoclast-mediated endocytosis and finally osteoclast apoptosis.[58] Bisphosphonate absorption by the body differs depending on the delivery method. When delivered orally, it is minimally absorbed in the intestines due to the hydrophilic nature of the compound, however, when delivered intravenously, it is 100% bioavalible.[58] This therapy is used to reduce fracture risks as

it inhibits bone remodelling, however, it may cause adverse side effects such as mild hypocalcaemia, uncharacteristic femoral fractures, gastrointestinal irritation, severe chronic musculoskeletal pain, ocular inflammation and osteonecrosis of the jaw.[56, 58]

Denosumab is a human monoclonal antibody used as an anti-resorptive agent which limits osteoclast-mediated resorption, however, this treatment is not yet available in South Africa.[58, 60] This antibody specifically binds to RANKL to prevent it from binding to RANK on osteoclast precursors thus disrupting osteoclast formation.[58] Denosumab is used to treat osteoporosis and also to reduce the risk of fractures in men who are undergoing androgen-therapy for non-metastatic prostate cancer as well as for women who are being treated for breast cancer using an additional aromatase inhibitor.[58, 60]

Teriparatide is a human recombinant PTH which, when injected subcutaneously once per day, it stimulates bone formation together with resorption, however, bone formation was found to increase more than resorption.[58] Treatment duration using teriparatide is limited to 24 months as it was thought to increase the possibility of developing osteosarcoma in a preclinical rat model.[58] Many of the Food and Drug Administration (FDA) approved drugs used to treat osteoporosis produce undesirable side effects in patients at a cost thus inexpensive and less harmful treatments are required to help ease the progression of osteoporosis.[58]

2.3 Mesenchymal stem cells (MSCs)

MSCs are multipotent adult stem cells which have the potential to differentiate into adipocyte, chondrocyte and osteoblast lineages[5, 62] (Figure 2.5).[63-71] MSCs can be isolated from bone marrow, adipose tissue and dental pulp and these lineages are characterised by the presence of certain surface markers such as cluster of differentiation (CD)73, CD90 and CD105.[62] MSCs express markers which differ depending on the source the cells were isolated from, hence it may express either embryonic stem cell or pluripotent markers.[5] Stem cells derived from bone marrow may express additional markers such as octamer-binding transcription factor 4 (Oct-4), Nanog, ALP and stage-specific embryonic antigen-4 (SSEA-4) while cells derived from adipose tissue express Oct-4, Nanog, sex determining region Y-box 2 (SOX-2), ALP and SSEA-4.[5]

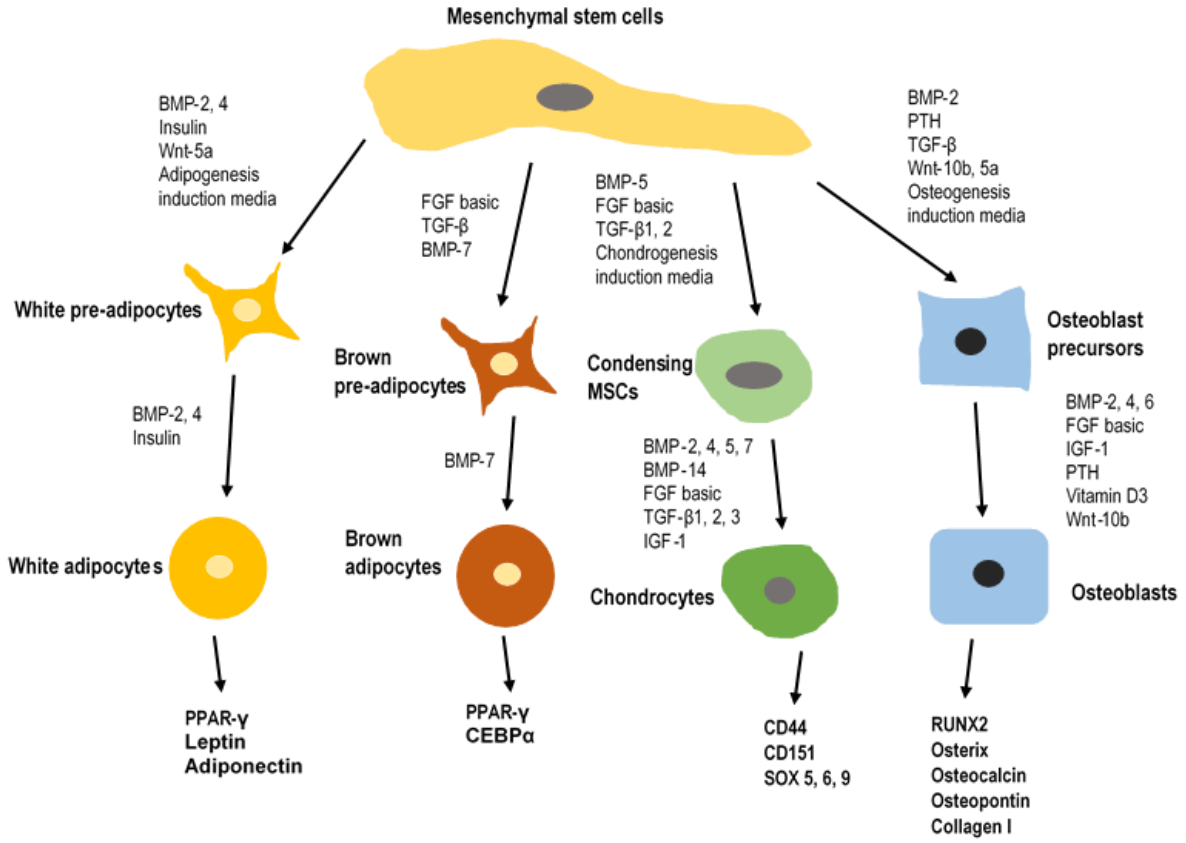

Figure 2.5. Mesenchymal stem cell differentiation.

There are multiple factors which influence the differentiation of MSCs into different lineages. White adipocytes express markers and proteins such as peroxisome proliferator-activated receptor γ (PPAR-γ), leptin and adiponectin while brown adipocytes which express PPAR-γ and CCAAT/enhancer binding protein alpha (CEBPα). Chondrocytes express CD44, CD151 and SOX -5, -6 and -9 after differentiation while osteoblasts express runt related transcription factor 2 (RUNX2), osterix (Osx), OCN, OPN and COL1 after differentiation. Image created from information by Chhabra *et al*.[63], Zhou *et al*.[64], Schäffler *et al*.[65], Takada *et al*.[66], Bennett *et al*.[67], Felber *et al*.[68], Rutkovskiy *et al*.[69], Narcisi *et al*.[70], and Terzoudis *et al*.[71]

MSCs are primary stromal cells which may be stimulated into osteoblast differentiation *in vitro* through supplementing culture medium with osteogenesis induction media containing ascorbic acid, β-glycerophosphate and dexamethasone[72] (Figure 2.5). Enzymes that hydroxylate proline and lysine in pro-collagen require ascorbic acid as a cofactor.[73] Ultimately, ascorbic acid plays a role in COL1 secretion into the ECM.[74] For osteoblast differentiation to occur, osteoblasts must be able to interact with the ECM containing collagen.[75] β-glycerophosphate is required as a source of phosphate in order to produce hydroxyapatite minerals ($Ca_{10}(PO_4)_6(OH)_2$). Inorganic phosphates regulate the gene expression of osteoblast markers such as OPN by acting as an intracellular signalling molecule which acts on extracellular signal-regulated kinase

(ERK).[76, 77] *In vitro*, MSCs undergo osteoblast differentiation, mineralisation and ultimately produce calcified nodules which resemble woven bone.[72] In a study by *Bellows et al*., it was found that β-glycerophosphate induced mineralisation which occurred in two phases. In the first initiation phase, a small number of minerals are deposited while in the second progression phase, mineralisation occurs at a constant rate.[78]

Mineralisation occurs in the presence of ALP as it hydrolyses organic phosphates in order to release free inorganic phosphates.[79] Bone sialoprotein (BSP), a non-collagenous protein, is expressed in bone and is thought to cause hydroxyapatite crystal formation in the matrix of bone.[80] BSP knockout mice were found to have a decreased body and long bone growth yet they displayed increased trabecular bone mass and low bone turnover.[81]

MSC differentiation is regulated by biological, chemical and physical factors which determines the different outcomes of MSC differentiation.[4] Biological factors include, micro-ribonucleic acids (miRNA), transcription factors and signalling pathways while physical and chemical factors include mechanical stimulation, radiation or a high fat diet. MiRNA are 22 nucleotide non-coding ribonucleic acids (RNA) of which many have been shown to affect MSC differentiation.[4] For instance, miR-204 exerts pro-adipogenic and anti-osteoblastic differentiation effects by inhibiting RUNX2 expression in MSCs.[4] MiR-31 similarly exerts anti-osteoblastic differentiation effects through inhibiting Osx expression during osteoblastogenesis.[4]

Transcription factors play a regulatory role in the differentiation of MSCs into adipocytes or osteoblasts. These factors upregulate gene expression for particular cell types.[4] RUNX2 is a significant transcription factor which plays a role in the formation and maintenance of bone.[82-84] RUNX2 binding elements are found in the promoter regions of osteoblast-specific genes such as Osx.[85-88] Osx is a novel zinc finger-containing transcription factor and is crucial for osteoblast differentiation from pre-osteoblasts.[89] Osx knockout mice showed normal cartilage development but they showed an absence of bone formation. These mice also had normal RUNX2 expression which indicates that Osx occurs downstream from RUNX2. Osx is thus essential for the differentiation of osteoblasts and bone formation in early bone development.[90]

There are a number of signalling pathways which demonstrate osteoblastic and adipogenic differentiation regulation in MSCs of which, two significant pathways are BMP and Wnt signalling pathways.[4] With a number of signalling pathways affecting MSC differentiation, there may be crosstalk among them thereby regulating osteoblastic and adipogenic differentiation.[4]

2.4 Pathways involved in osteoblastogenesis

As previously mentioned, RUNX2 serves as an important transcription factor in osteoblast differentiation as several signalling pathways intersect to express RUNX2 such as the BMP, Wnt, MAPK and Hedgehog (Hh) signalling pathways.[91]

2.4.1 Bone morphogenic protein (BMP) pathway

The BMP pathway is involved in regulating the differentiation of MSCs and osteoprogenitors into chondrocytes and osteoblasts.[5] BMPs form part of the TGF-β family of cytokines.[92] BMP receptors (BMPR) are serine-threonine kinase receptors, of which there are two subtypes, namely BMPR-I and BMPR-II. These receptors are formed on the plasma membrane of the cell and are recruited together to form a complex.[93] The signal is transduced once the activated receptor kinases phosphorylate Smad family member 1 (Smad1), 5 or 8 transcription factors. Smads are divided into receptor regulatory Smads (R-Smads), inhibitory Smads (I-Smads) and common Smads (Co-Smads).[94] There are seven Smad members which have been known to have ligand selectivity.[95] Smad1 has been associated with BMP responses while Smad2/3 are activated after TGF-β treatment.[95] Smads1/5/8 are R-Smads and once phosphorylated, bind to Smad4 which is a Co-Smad, to enter the nucleus and activate the transcription of target genes (Figure 2.6).[4, 94, 96-98] BMPs are found in bone ECM and are secreted by osteoprogenitors, MSCs, osteoblasts and chondrocytes to target osteoblasts.[5]

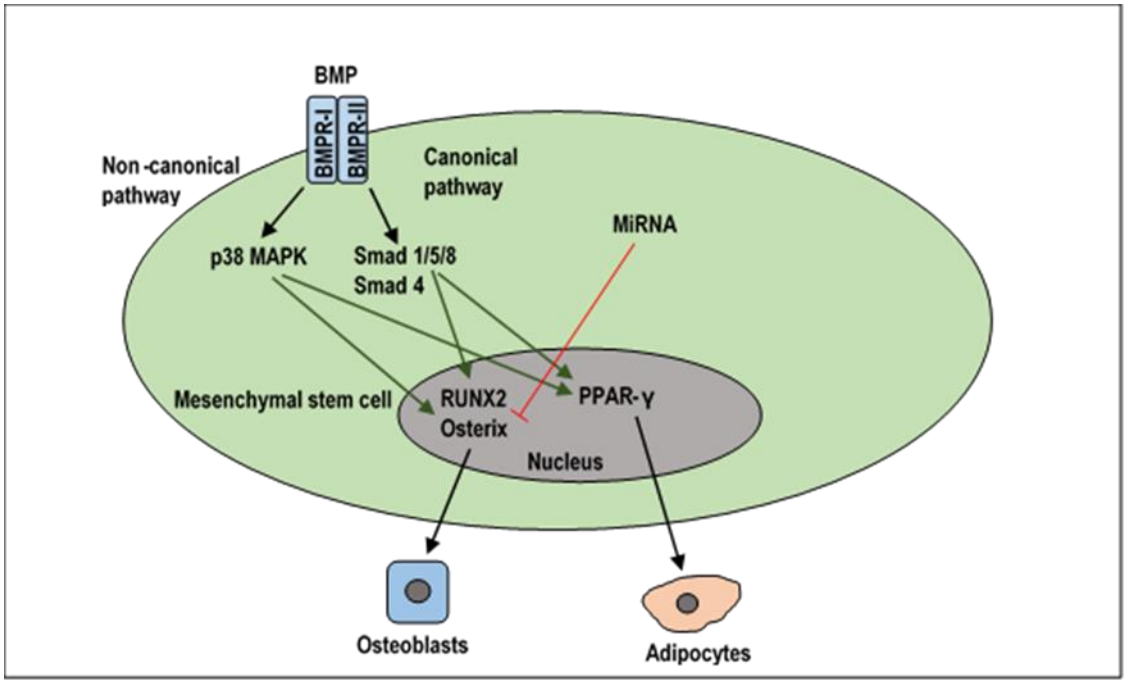

Figure 2.6 BMP signalling can occur through a canonical or non-canonical pathway.
BMP ligands bind to BMPR-I/BMPR-II complex to either induce the non-canonical p38 MAPK pathway or the canonical pathway through the phosphorylation of Smads, which acts as a transcription factor to induce RUNX2/Osx activation in the nucleus. MiRNAs may target important transcription factors to inhibit RUNX2/Osx transcription. Image created from information by Hu *et al*.[4]

TGF-β is a chemotactic stimulator of MSCs which enhances the proliferation of MSCs, pre-osteoblasts, chondrocytes and osteoblasts. TGF-β initiates BMP signalling in osteoprogenitor cells, inhibits osteoclast activation and causes osteoclast apoptosis.[5] Conversely, TGF-β1 may furthermore induce non-Smad signalling through MAPK pathway activation.[98] Without TGF-β and BMP-2, normal fracture healing does not occur as MSCs will not differentiate into an osteogenic lineage.[5] RUNX2 is a target for the BMP-Smad pathway (Figure 2.6), as treatment with BMP-2 increases the protein levels of ALP and RUNX2 gene expression.[4, 99] Osteoblast-specific genes such as COL1 and OCN could be regulated by RUNX2 which is involved in the regulation of MSCs differentiation into osteoblasts.[85-88] Its expression initiates bone mineralisation and increases the expression of osteoblast-specific genes.[87, 88] RUNX2 knockout mice demonstrated a diminished number of functional differentiated osteoblasts as a result of a lack in RUNX2 expression.[100] Furthermore, RUNX2 overexpression was found to favour osteogenesis over adipogenesis, hence RUNX2 could increase bone healing efficiency.[101, 102]

The BMP pathway interacts with other factors which affect MSCs differentiation into osteoblasts (Figure 2.7).[5] Osteogenic differentiation in human MSCs is additionally stimulated by cyclin dependent kinase 1 (CDK1) through the phosphorylation of enhancer of zeste homolog 2 (EZH2) which is known to regulate stem cell differentiation. CDK1 knockdown has been shown to lead to a decrease in the phosphorylation of EZH2 and the repression of RUNX2 and OPN.[5] Stromal cell-derived factor 1 (SDF-1) has also been known to stimulate MSC differentiation into osteoblasts. Culturing cells in both OM and SDF-1 showed increased ALP activity compared to culturing cells in OM only.[103] Disrupting SDF-1 signalling resulted in impaired mineralization of bone nodules and inhibited BMP-2 induced early expression of RUNX2 and Osx.[104] SDF-1 is a chemoattractant for the migration of MSCs to injury sites where it is upregulated and activates the C-X-C chemokine receptor type 4 (CXCR4) receptor.[105, 106] SDF-1 and CXCR4 combine to form a complex that promotes growth and metabolic activity at injury sites.[105]

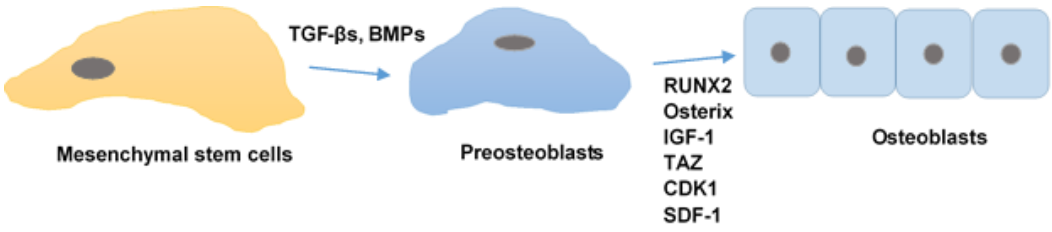

Figure 2.7 Factors which affect MSC differentiation into osteoblasts.
TGF-β and BMP-2 are essential for normal fracture healing to occur and influences RUNX2 activation downstream in the BMP and Wnt pathways. CDK1 and SDF-1 also play a role in MSC differentiation and migration. Tafazzin (TAZ), a RUNX2 transcriptional co-activator of OCN, promotes MSCs differentiation and inhibits PPAR-γ from stimulating MSCs adipocyte differentiation. Image created from information by Garg *et al*.[5]

2.4.2 Wingless-related integration site (Wnt) signalling pathway

Osteoblastogenesis is controlled by a second pathway, the Wnt signalling pathway. Wnts form a part of a group of secreted cysteine-rich glycoproteins that play a role in cellular migration, proliferation and differentiation.[107] Signals can be transduced via a canonical or non-canonical pathway.

In the canonical pathway, β-catenin is compulsory for signalling whereas in the non-canonical pathway, β-catenin is not compulsory.[108] Extracellular Wnts bind to the expressed Frizzled (Fzd) transmembrane receptor as well as to the co-receptor low-density lipoprotein receptor-related protein (LRP) 5/6 and proteins part of the dishevelled family, illustrated in Figure 2.8.[4, 107] This complex inhibits the axin, adenomatosis polyposis coli (APC) and glycogen synthase kinase 3β (GSK3β) protein complex which in turn prevents the phosphorylation of β-catenin, preventing it from being degraded.[107] An increase in β-catenin allows it to enter the nucleus and form a complex with the T-cell factor/lymphoid enhancer factor (TCF/LRF). This leads to β-catenin accumulating in the nucleus and stimulates the transcription of target genes.[109]

The non-canonical pathway activates heterotrimeric G proteins and also increases calcium intracellularly, illustrated in Figure 2.8.[107, 110] Intracellular release of calcium regulates calcium-sensitive enzyme activity and causes downstream targets to be activated such as transforming growth factor beta-activated kinase 1 (TAK-1)/ TAK1-binding protein 2 (TAB-2) thus inhibiting the transcription of target genes (Figure 2.8).[107] This could occur through protein kinase C-dependent mechanisms or it could be induced via rho-kinase or *c-Jun* N-terminal kinase (JNK)-dependent changes which mediate cytoskeleton formation.[110]

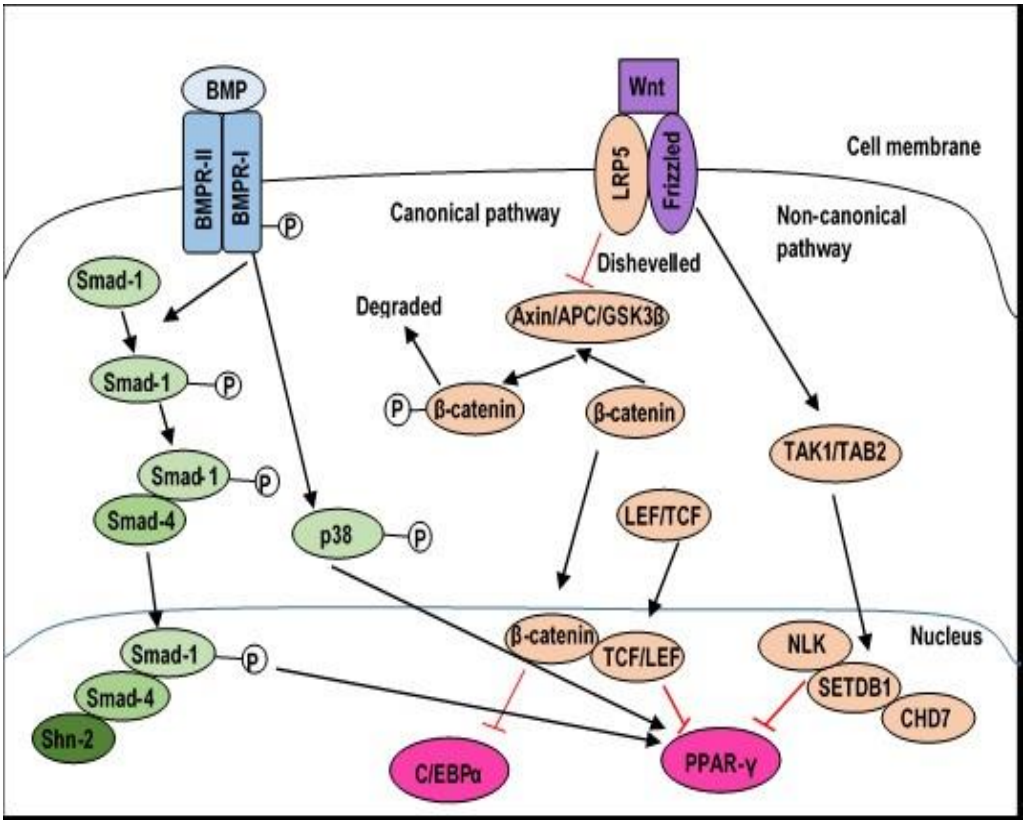

Figure 2.8. Osteogenesis and adipogenesis regulation by BMP and Wnt signalling pathways. BMPR-I phosphorylation initiates the phosphorylation of Smad and p38 MAPK pathways. The phosphorylation of p38 and Smad ultimately stimulates PPAR-γ activation. This ultimately promotes adipogenesis. Wnt/Fzd complex stimulates canonical and non-canonical signals. Canonical signals stabilize β-catenin to suppress PPAR-γ. Non-canonical signals (TAK-1/TAB-2) to suppress PPAR-γ transactivation to promote osteogenesis. Image created from information by Muruganandan *et al*.[107]

2.4.3 Mitogen-activated protein kinase (MAPK) pathway

MAPK plays an essential role in skeletal development as well as in bone homeostasis which affects osteoblast differentiation.[111] MAPK are a group of enzymes that contain a serine-threonine kinase domain which transduce extracellular signal into cellular actions by a series of phosphorylation. Different signalling pathways may also converge to activate MAPK.[111] There are five conventional MAPK namely ERK1/2, ERK5, JNK1/2/3, and p38, of which there are four isoforms (p38α, -β, -γ and -δ).[111] MAPK activates and phosphorylates RUNX2 to stimulate osteoblast differentiation by stimulating the transcription of osteoblast markers such as OCN.[75]

ERK1/2 is expressed by osteoblasts and plays a role in bone metabolism. Moreover, ERK1/2 has a positive effect on osteoblast differentiation as it is required for RANKL

expression in osteoblasts and for osteocalcin-expressing mature osteoblasts.[112] Inactivation of ERK1/2 reduces RANKL which impacts osteoclast differentiation.[112] Hu *et al*., investigated the role of ERK in osteoblast differentiation. Inhibition of ERK had no significant effect on cell differentiation, however, at an early stage of osteoblast differentiation, ERK activation was observed.[47] In another study, Lai *et al*., demonstrated that dominant negative (a gene product that acts as an antagonist to the wild-type gene product) ERK1 inhibited human osteoblast differentiation as well as proliferation and migration.[113] This discrepancy could be due to the source of the cells as Hu *et al*., used primary calvarial osteoblasts and mouse bone marrow stromal cultures while Lai *et al*., used human bone chips. Furthermore, ERK1 was inhibited in different manners. In the study by Hu *et al*., an inhibitor of ERK1 was used while in the study by Lai *et al*., a dominant negative form of ERK1 was used which could also contribute to the differences observed.[47] Moreover, Hu *et al*., indicated that ERKs may negatively affect osteoblast differentiation induced by BMP-2.[47]

P38 MAPK coding genes display a unique tissue distribution and appear to be differentially expressed.[111] p38 MAPK activation has been demonstrated to interfere with osteoblast differentiation.[47] Once p38 MAPK was inhibited, the differentiation markers for ALP, OCN and mineral deposition were decreased, thus suggesting p38 MAPK is required for osteoblast differentiation at an early stage.[47] Osteoblast differentiation was found to be delayed by the inhibition of p38 MAPK which, was partially released by the presence of BMP-2. This suggests that the loss of signals from p38 MAPK can be overcome by BMP-2 during osteoblastogenesis.[47] Yoshida *et al*., indicated that through the inhibition of Smad proteins, BMPs may be able to control osteoblast differentiation. Hence, by activating the BMP-Smad pathway, the loss of p38 MAPK may be counteracted.[94] It is hypothesized that p38 MAPK exerts its effect either by directly or indirectly phosphorylating transcription factors through activating other kinases which consequently phosphorylates its targets downstream. This could in turn increase the deoxyribonucleic acid (DNA) binding affinity or activate their trans-activation ability to upregulate genes involved in differentiation.[47] TAK-1/TGF-β-activated protein kinase 1 (TAB-1) facilitates the recruitment and activation of upstream effectors of p38 which may also be controlled by additional mechanisms such as acetylation, protein degradation and changes in coding gene expression.[111]

JNK consists of three isomers and forms part of the MAPK pathway to phosphorylate *c-Jun* in its N-terminal trans-activation domain.[114] A study by Matsuguchi *et al*., established the role of JNK in osteoblasts.[114] Results showed JNK inactivation significantly inhibited terminal osteoblast differentiation and also supressed late stage osteoblast differentiation events including OCN and BSP gene expression.[114] Earlier differentiation events such as ALP and OPN gene expression were not supressed by the inactivation of JNK.[114] Transcriptional expression of factors such as RUNX2 and Osx were not significantly affected by JNK inactivation. The study concluded that JNK activity is crucial for late-stage osteoblast differentiation.[114] Figure 2.9 shows how BMP-2 can stimulate osteoblast differentiation through RUNX2, however, RUNX2 can also be inhibited by tumour necrosis factor α (TNF-α) and interleukin 1β (IL-1β) alone or in conjunction with BMP-2.[115] Therefore, in an inflammatory environment, BMP-2 induced activation of RUNX2 may be reduced, inhibiting osteoblast differentiation.[115]

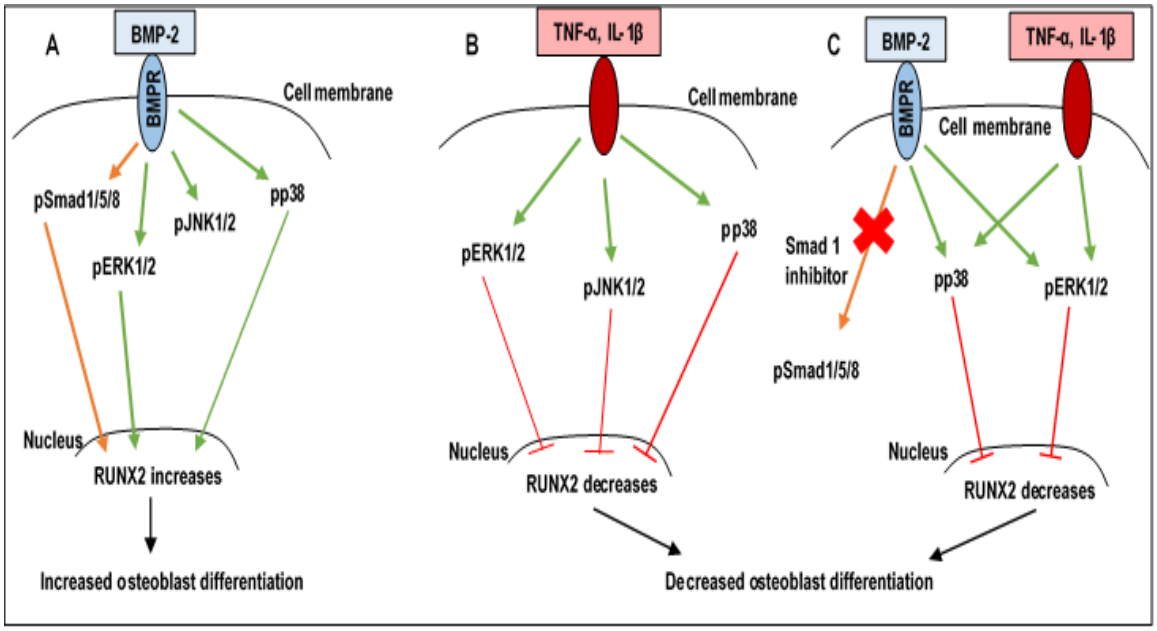

Figure 2.9. BMP-2 activates BMPR-I/II to stimulate the canonical and non-canonical BMP pathways in order to stimulate osteoblast differentiation through MAPK.

BMP-2 activation of BMPRI/II can be opposed by TNF-α/IL-1β activation. (**A**) BMP-2 pathway activation stimulates the MAPK pathway to phosphorylate p38, ERK1/2 and JNK1/2 which, in turn increases RUNX2 expression and thus stimulates osteoblast differentiation. (**B**) TNF-α and IL-1β activates MAPK pathways through p38, ERK1/2 and JNK1/2 but it has an opposite effect and thus reduces RUNX2 expression. (**C**) Activation of BMP-2 and TNF-α/IL-1β reduces RUNX2 expression through the activation of MAPK. Image created from information by Huang *et al*.[115]

2.4.4 Other pathways affecting osteoblastogenesis

Other pathways also affect osteogenesis. During postnatal growth, the growth hormone (GH) signalling pathway is activated as GH binds to GH receptor (GHR), ultimately activating the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway amongst others. This leads to growth and metabolism of tissues such as bone.[98] In mice, osteoblastogenesis can be stimulated through RUNX2 interaction in the Hh signalling pathway. Hh signalling is essential for skeletal patterning throughout development.[116, 117] Wnt signalling occurs downstream of Hh.[118] Osteogenesis induced by Hh signalling correlated with increased RUNX2 and Osx expression.[118] There are three homologs of Hh, two of which are Sonic hedgehog (Shh) and Indian hedgehog (Ihh) and they are both involved in the development of osteoblasts.[119] In human MSCs, Hh signalling decreases RUNX2 expression during osteoblast differentiation and thus inhibits osteogenesis in contrast to rodent cells.[119] The inhibition of nuclear factor kappa-B (NF-κB) inflammatory pathways in murine osteoblasts expressing a dominant-negative form of nuclear factor kappa-B kinase subunit beta (IKKβ), showed an increase in BMD and an increased bone volume due to increased osteoblast activity. NF-κB inhibition also enhanced BMP-induced osteoblastogenesis and bone formation.[120]

2.5 Adipose-derived stromal cells

ASCs are a type of adherent multipotent cell population.[6] The capacity of MSCs to differentiate into adipocytes provides a model to study adipogenesis. Adipocytes are perceived as complex endocrine cells which affect homeostasis in the body and can secrete cytokines, chemokines and hormones.[121] Adipocytes express CD36 on its cell surface. Its function is to facilitate the uptake of free fatty acids (FFA) into cells which is followed by esterification into triglycerides where they are subsequently stored within lipid droplets.[121] ASCs express CD13, CD29, CD36, CD44, CD73, CD90 as well as CD105 surface antigen markers, and are converted into preadipocytes and subsequently become mature adipocytes through Wnt, BMP-2 and -4 signalling pathways.[6] Adipogenesis and osteogenesis pathways are linked as they both stem from MSCs[71] (Figure 2.5). Increases in adipocyte formation and the accumulation of fat may cause decreases in osteoblast differentiation and bone formation.[6]

2.5.1 Adipogenesis

White adipose tissue store triglycerides and is involved in lipogenesis (metabolic formation of fat) and lipolysis (the breakdown of fats), and the activity of these mechanisms depends on the need to store or release FA.[122] The body's need for FA depends on the individual's energy expenditure, levels of hormones and enzyme activities.[122] In a recent study, adipocytes were found to release factors such as palmitic acid (PA, $C_{16}H_{32}O_2$), a saturated FA which inhibits osteoblast differentiation by causing apoptosis in bone marrow MSCs.[123] Adipogenesis may be inhibited while osteoblastogenesis may be induced through Hh signalling.[124] PPAR-γ is supressed during osteogenesis by IL-1 and TNF-α.[125] The mechanism of PPAR-γ suppression by IL-1 and TNF-α differs from the mechanism induced by non-canonical Wnt ligands.[124] IL-1 and TNF-α impedes adipogenesis through the TAK-1/TAB-1 pathway which in turn suppresses PPAR-γ (Figure 2.8).[107, 124] Non-canonical Wnt5a activates nemo-like kinase (NLK) causing the formation of PPAR-γ/SET domain containing 1 (SETD1) complex (Figure 2.8).[107] This leads to transcriptional silencing thereby inhibiting PPAR-γ-induced adipogenesis.[124]

Adipogenesis may be regulated by PPAR-γ, a receptor found on the nucleus, as its suppression has been demonstrated to prevent adipogenesis and subsequently induce osteoblastogenesis.[124, 126] Canonical Wnt signalling similarly has been shown to affect osteoblastogenesis and adipogenesis through LRP5/β-catenin.[127, 128] Wnt and BMP-2 signalling pathways integrate at various points such as at the expression of RUNX2 and β-catenin.[91] The differentiation of MSCs into adipocytes is stimulated at low levels of BMP.[5] Thus Wnt signalling plays a crucial role in the regulation of PPAR-γ transactivation in MSCs, as seen in previous studies, and may also determine the fate of MSCs.[6, 124, 128]

2.5.2 The role of peroxisome proliferator-activated receptors (PPARs) in adipogenesis

PPARs are a receptor group belonging to the steroid hormone receptor superfamily.[129] This receptor group has been implicated in adipogenesis. PPARs are involved in pathways for fat storage and lipid metabolism.[130, 131] There are three PPAR receptor subtypes, PPAR- α, β and γ. PPAR-α is found mainly in the liver while PPAR-β is

highly expressed in skin, brain and adipose tissue.[132, 133] Both of these subtypes have been found to be expressed in osteoclasts and osteoblasts.[134] PPAR-γ is the third nuclear receptor subtype, which is expressed in adipocytes and pancreatic beta cells.[129, 133]

PPAR-γ has two isoforms which arise due to alternative splicing and promoter usage. PPAR-γ1 is expressed by adipocytes, muscles, macrophages, osteoclasts and osteoblasts. PPAR-γ2 is expressed only in adipocytes and is an essential transcription factor in MSCs lineage commitment.[132, 135, 136] Additionally, PPAR-γ is essential for adipocyte gene expression.[129] Moreover, it stimulates stem cell differentiation towards adipocytes instead of osteoblasts.[137, 138] PPAR-γ overexpression has previously been shown to suppress RUNX2 expression and ultimately promote the formation of adipocytes.[138] During age-related osteoporosis, a decline in bone mass is found to be accompanied by an increase in marrow adipose tissue formation.[139] PPAR-γ transcriptional activity may be modulated through negative and positive interactions with other signalling pathways.[124] In addition to this, PPAR-γ may be activated by FFA.[140] The phosphorylation of PPAR-γ at the N-terminal domain is known to supress the activation of PPAR-γ through decreasing its affinity for PPAR-γ ligands. PPAR-γ ligand-dependent post-translational modification which incorporates small ubiquitin-like modifier proteins, represses NF-κB and thus antagonises inflammatory responses.[124] BMP signalling molecules act as osteogenic inducers and may impact the differentiation of adipocytes via the induction of TAZ, a PPAR-γ co-repressor.[124] Furthermore, TAZ is a transcriptional co-activator for OCN and RUNX2.[141] The upregulation of OCN may correlate with increased osteoblast proliferation.[141]

2.6 Fatty acids

FA comprise of hydrocarbon chains with a terminal carboxyl group at one end and a methyl group at the other end. FA may be saturated in which they contain no double bonds, or monounsaturated when they contain only one double bond, or polyunsaturated when they contain two or more double bonds in their carbon chain (Figure 2.10).[142, 143] UFA can have two geometric configurations, they may be of *cis* configuration where two hydrogen atoms are found on the same side of the carbons joined by a double bond or they may be in *trans* configuration where the hydrogen atoms are found on the opposite sides of the carbons joined by a double bond.[142]

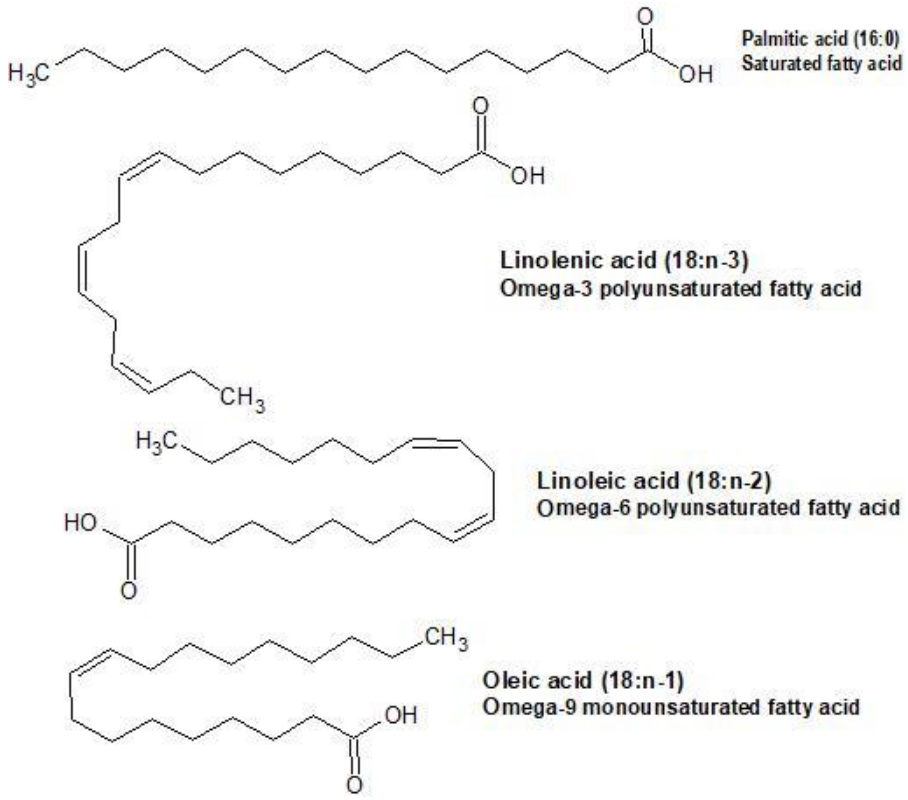

Figure 2.10. Fatty acids may be saturated, monounsaturated or polyunsaturated.

Fatty acids contain a terminal carboxyl group (-COOH) at one end and a methyl group (-CH_3) on the other end. They may have none, one, two or three double bonds within their carbon chain. Omega -3, -6 or -9 polyunsaturated fatty acids refer to the first double bond being at positions -3, -6 or -9 from the methyl group. Image created from information by Dandekar *et al*.[143]

2.6.1 Types of fatty acids and their health benefits

Saturated fatty acids

SFA have no double bonds in their carbon chains. PA (16:0) (Figure 2.10), stearic acid (SA; 18:0), myristic acid (MA; 14;0) and lauric acid (12:0) are abundant saturated FA found in the diet.[144] Sources of SFA include palm oil, coconut oil, cocoa butter and animal-derived fats for example lard and butter.[145] SFA may also be synthesized *de novo* in humans from acetyl-CoA.[145] SFA such as lauric acid, PA and MA may increase total and low-density lipoprotein (LDL) cholesterol. Evidence from human studies suggests there is a positive association between the intake of dietary SFA and insulin resistance.[146] Circulating concentrations of even carbon atom numbered FA such as MA, PA and SA were positively associated to the incidence of type 2 diabetes while odd numbered FA such as pentadecanoic acid (C15:0) and heptadecanoic acid (C17:0) were inversely associated with the incidence of type 2 diabetes.[147]

Polyunsaturated fatty acids

PUFA include omega-3 and -6 FA. Omega-3 PUFA contain a double bond at position 3 from the methyl end while omega-6 PUFA contain a double bond at position 6 from the methyl end (-CH3) (Figure 2.10).[143] PUFA can arise from either the linoleic acid (LA; 18:2n-6) family, which can have a double bond at positions 6 or 9 from the terminal methyl group, or the α-linolenic acid (ALA; 18:3n-3) family which can have a double bond at positions 3, 6 or 9 from the terminal methyl group (Figure 2.10).[148]

ALA desaturates and elongates to form omega-3 PUFA.[149] ALA is mainly found in plants and more specifically in seeds, nuts and some vegetable oils such as flaxseed and walnut oils.[149] It is the parent chain of both eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). ALA can be used to synthesize EPA and DHA by the body, however, the bioconversion is inadequate due to the limitation in the enzyme responsible for this conversion thus it must be supplemented by our diet.[149] Certain fish such as salmon, herring and sardine are dietary sources of DHA and EPA.[149] In a 2007 Japanese study, termed the JELIS study, highly purified EPA administered together with statin resulted in a significant prevention in cardiovascular events through a cholesterol-independent mechanism even in patients who had recently experienced a myocardial infarction.[150]

Omega-3 PUFA may exert their anti-inflammatory properties through modifying cell membranes which are composed of phospholipids.[151] These phospholipids contain FA of which their length and saturation alters the properties of the cell membrane. The incorporation of omega-3 PUFA into phospholipids can influence the function of membrane proteins such as the suppression of IL-2. This may cause the modulation of ion channel function, resulting in anti-arrhythmic effects.[151] The anti-inflammatory effects of omega-3 PUFA may also be mediated by receptors such as G-protein coupled receptors (GPCR) such as GPR120.[151] Omega-3 PUFA are ligands for GPR120 receptors which reduces TNF-α-mediated pro-inflammatory signalling in macrophages. GPR120 is also known to be expressed in mature adipocytes and stimulation of this receptor with omega-3 PUFA promotes glucose uptake in adipocytes through increasing the expression of the GLUT4 glucose transporter.[151] Moreover, EPA and DHA inhibited NF-κB signalling either through blocking inhibitor of

nuclear factor κB (IκB) phosphorylation or through PPAR-α/γ which ultimately downregulated inflammation-related gene expression.[151]

Omega-6 FA such as arachidonic acid (AA; 20:4n-6) are synthesized from LA. A primary source of LA is vegetable oils such as corn, sunflower and soybean oils.[149] LA is known as an essential FA as it cannot be synthesized by humans.[152] LA can be desaturated to form other omega-6 FA such as γ-linoleic acid (GLA; 18:3n-6), which then undergoes elongation to form dihomo-γ-linoleic acid (DGLA; 20:3n-6), which can then be desaturated to form AA, which can be further metabolised into other omega-6 PUFA.[152] In a Western diet, AA is a major contributor of FA found in phospholipid membranes of cells involved in inflammation and is also a precursor to potent pro-inflammatory mediators.[152] ALA levels also tend to be lower in the diet than LA thus levels of omega-6 PUFA tend to be higher than omega-3 PUFA in the body.[149] A high omega-6 and low omega-3 PUFA intake is common in Western diets.[153]

Omega-3 and -6 PUFA are enzymatically converted into eicosanoids which are hormone-like compounds that have a chain length of 20 carbons. Eicosanoids are produced from precursor FA such as AA, EPA and DGLA when PUFA are liberated from membrane phospholipids by phospholipases. These PUFA serve as a substrate for cyclooxygenases, lipoxygenases or cytochrome P450 monooxygenases.[154] Cyclooxygenases generate prostaglandins and thromboxane's while lipoxygenases form hydroxy fatty acids, leukotrienes and lipoxins. Cytochrome P450 monooxygenases produce hydroxy fatty acids, epoxy fatty acids and dihydroxy fatty acids.[154] Eicosanoids are short-lived but biologically powerful compounds which can modulate inflammation and immune responses as well as play a role in platelet aggregation, cell differentiation and growth.[154]

Thromboxane A_2, formed from AA, is a potent vasoconstrictor and platelet aggregator. On the other hand, thromboxane A_3, formed from EPA, is a less potent vasoconstrictor.[155] Vascular endothelial tissue lacks thromboxane synthetase, however, expresses prostacyclin synthetase which causes the production of prostacyclin (PGI_2) from AA. PGI_2, a potent vasodilator, inhibits platelet aggregation. PGI_3 formed from EPA, however, is a less potent vasodilator and has weaker platelet aggregation properties.[155] Therefore, eicosanoids produced from AA generally exhibits pro-inflammatory properties while eicosanoids produced from EPA exhibit

anti-inflammatory properties.[154] In a study by Uauy *et al*., DHA and EPA reduced the clinical risk of developing cardiovascular disease due to the alteration of the duration of bleeding and platelet aggregation.[149, 156] In a review article, Shahidi *et al*., summarised numerous research findings on the effects of omega-3 PUFA on clinical trials in cardiovascular health.[149]

Monounsaturated fatty acids

MUFA have higher melting points than PUFA and only contain one double bond in their carbon chains. PLA (16:1n-7) an omega-7 MUFA (Figure 2.11) and oleic acid (OA; 18:1n-9) an omega-9 MUFA (Figure 2.10) are two common MUFA which occur in *cis* conformation.[143, 157] MUFA can be found in numerous vegetable oils such as canola and olive oil, various nuts and seeds such as cashews and peanuts, and in avocado, olives and certain meats.[158] OA can also be synthesized by mammals, including humans.[153] High levels of MUFA are found in Mediterranean diets.[159] A traditional Mediterranean diet consists of minimally processed whole grains and legumes; a large variety of fresh vegetables consumed daily; fresh fruits; a majority source of fats from nuts, seeds and olive oil; moderate fish consumption; low amounts and frequency of dairy and red meat intake; and low to moderate wine consumption.[159]

Recently, in a randomized clinical trial, individuals on a Mediterranean diet were supplemented with extra-virgin olive oil and found to have a significant decrease in inflammatory markers as well as circulating oxidised LDL.[159] It has been reported that the Mediterranean diet protects against inflammation, oxidative stress, and platelet aggregation; and the modification of growth factors and hormones involved in cancer pathogenesis.[159, 160] Dietary MUFA intake is also associated with a healthy blood lipid profile, mediates blood pressure, and modulates insulin sensitivity as well as glycaemic control which can ultimately reduce the risks of developing cardiovascular disease.[11, 159, 160] In addition, studies suggested that metabolic syndrome and even cognitive impairments such as Alzheimer's disease can be prevented and reduced by following a Mediterranean diet.[160-162]

PA is a saturated FA containing 16 carbons (Figure 2.11).[163] It undergoes dehydrogenation to form PLA which is a monounsaturated omega-7 FA. PLA has a *cis* double bond at the seventh carbon from the methyl end.[12, 164] Dietary sources high in PLA include eggs, macadamia nuts, chocolate, olive oil, salmon, cod liver oil and

sea buckthorn.[164] The biosynthesis of PLA takes place mainly in the liver but may also take place in adipose tissue where it can then be incorporated into phospholipids.[164] PLA is synthesized endogenously by adipocytes through the desaturation of PA by stearoyl-CoA desaturase (SCD-16). PLA synthesis results from lipogenesis in humans and is more abundant in abdominal subcutaneous adipose tissue.[12, 163, 164] Furthermore, PLA is considered a novel lipokine as is it release by adipose tissue but exerts an effect on distant organs.[164]

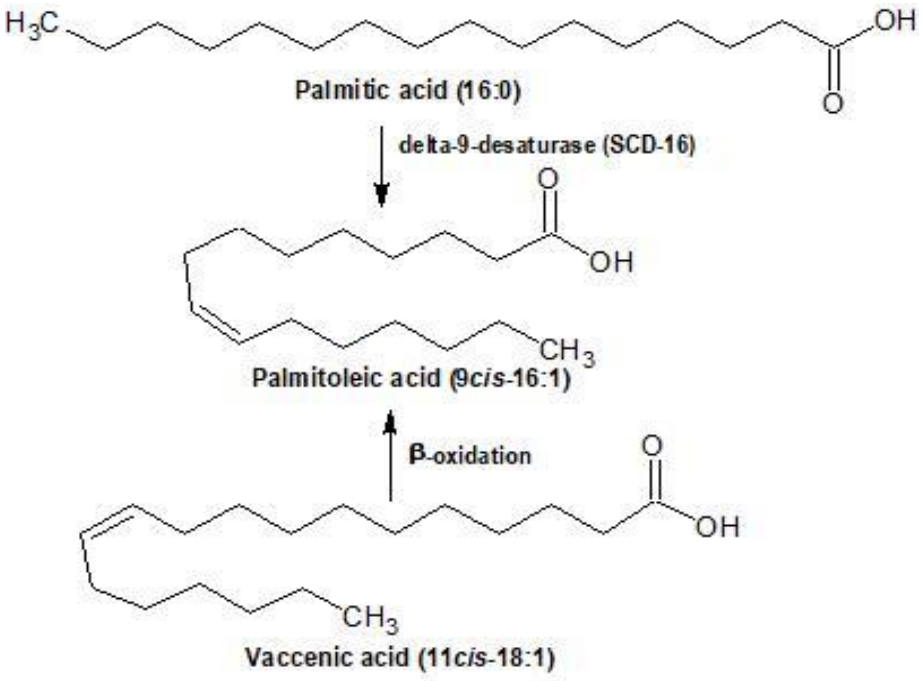

Figure 2.11 Palmitic acid undergoes dehydrogenation to form palmitoleic acid.
Dehydrogenation of PA can occur via SCD-16 or via beta-oxidation of vaccenic acid, a *trans* fatty acid found in human milk and dairy products, to form PLA. Image created from information by Sansone *et al*.[163]

Bolsoni-Lopes *et al*., demonstrated that the binding affinity of PPAR-α to peroxisome proliferator-responsive element (PPRE), its DNA consensus sequence, increased significantly after 12 hours of PLA treatment. This indicates that PPAR-α is activated by PLA, however, it is unknown whether PLA acts as a direct PPAR-α ligand. Further studies are required to establish the mechanism of action.[165] Mice treated with PLA demonstrated a significant increase in adipocyte lipolysis while mice fed on PA rich high fat diet showed a reduction in adipose tissue *de novo* fatty acid synthesis as well as PLA content. This demonstrated that PLA may contribute towards important metabolic processes in adipose tissue such as adipocyte lipolysis.[165] In animal

models, *cis*-PLA resulted in a reduction in the expression of pro-inflammatory markers and adipokines.[164] However, in humans, the role of PLA in the development of obesity and its influence on liver and cardiovascular health may not be clear. A high concentration of PLA was associated with a decrease in the prevalence of diabetes.[164]

PLA has been associated with suppressing the expression of adipocyte cytokines, promoting pancreatic β-cell proliferation as well as its secretary functions, enhancing glucose uptake in skeletal muscle, and stimulating the transcriptional activity of PPAR-γ.[166] In a study by Mozaffarian *et al*., plasma phospholipid PLA was independently associated with favourable high-density lipoprotein (HDL) cholesterol as well as total:HDL cholesterol, thus potentially supporting the metabolic benefits established in animal models.[166] However, it was also associated with increased adiposity and triglyceride levels as well as an increase in insulin resistance in men.[166] In contrast, a study by Nestel *et al*., reported that PLA significantly lowered HDL cholesterol in comparison to PA in hypercholesteraemic men.[167] Thus, studies have demonstrated conflicting results on the effects of PLA on HDL cholesterol and more work is required to establish the effects of PLA on HDL cholesterol.

A more recent study by Souza *et al*., described the anti-inflammatory effect of PLA on endothelial cells.[168] The study concluded that PLA reduced cytokine and adhesion molecule production while downregulating pro-inflammatory gene expression for NF-κB, IL-6 and cyclooxygenase-2, and upregulating PPAR-α gene expression.[168] PA increased the concentration of AA, a pro-inflammatory FA, while PLA increased the concentration of vaccenic acid, an anti-inflammatory FA, thus suggesting a protective effect against cardiovascular disease.[168] Several studies have thus shown the beneficial effects of PLA on metabolic processes, cardiovascular health and inflammation.[164, 166-169]

2.7 Fatty acids and bone

There are numerous dietary and other factors which are known to affect bone health. These include calcium, PTH, vitamin D, phosphorus, magnesium and potassium which enhance bone health.[170] An individual's nutritional requirement changes during their lifespan and if not adequate, could result in conditions such as osteopenia and osteoporosis.[170] PUFA and MUFA have shown promising results in preventing and

reducing the effects of metabolic bone conditions *in vivo* and *in vitro*, however, their exact mechanisms are currently not fully understood.[171]

2.7.1 *In vitro* studies

Several *in vitro* studies have been considered in examining the link between bone and FA. Studies of osteoblasts and osteoclasts have mainly focussed on the effects of different FA on cells involved in bone formation, bone resorption as well as the production of pro-inflammatory or anti-inflammatory cytokines.[14, 172] Higher levels of soybean, sunflower and corn oils in the diet have been known to inhibit osteogenesis through the expression of PPAR-γ in bone cells and thus promoting the differentiation of MSCs into adipocytes instead of osteoblasts.[173]

Saturated and unsaturated FA may act as signal molecules. SFA could produce reactive oxygen species (ROS) and accumulation of this leads to lipotoxicity in cells, ultimately leading to cellular dysfunction and death.[14] PUFA such as AA, ALA and LA are natural ligands for PPAR-γ. The effects of compounds derived from the metabolism of omega-3 FA have demonstrated weaker effects on MSC differentiation into adipocytes than omega-6 FA.[14] The activation of PPAR-γ2 by these metabolised PUFA leads to a reduction in osteoblast production as a result of an increase in adipocyte formation in MSCs.[14] Other UFA such as DHA, EPA, PLA and OA have been shown to modulate the activity of osteoblasts and osteoclasts though free fatty acid receptor 4 *in vitro*.[9]

2.7.1.1 Osteoblast studies

In vitro studies have been conducted on the effects of SFA, such as palmitate, on bone cells. Palmitate, an ester of PA, has shown to have cytotoxic effects on human MSCs and osteoblasts derived from MSCs through inducing endoplasmic reticulum stress and activating NF-κB as well as ERK pathways.[172] Cells exposed to PA produced pro-inflammatory responses, with osteoblasts derived from MSCs displaying more sensitive lipotoxicity than undifferentiated MSCs.[172] The presence of OA along with PA, on the other hand, counteracted the pathways which PA had activated. This may be due to OA inducing the esterification of PA into triglycerides and lipid droplet storage.[172] Studies on the effects of palmitate on osteogenesis in human cells also

demonstrated a reduction in transcriptional activity of RUNX2 and β-catenin thus negatively affecting bone formation.[172]

Other studies have reported on the effects of PUFA on osteoblasts. Watkins *et al*., described the modulatory effects of omega-3 PUFA on osteoblast function using MC3T3-E1 pre-osteoblasts *in vitro* and reported that omega-3 PUFA increased the expression of ALP activity and reduced prostaglandin E_2 (PGE_2) production thus possibly promoting bone formation by osteoblasts.[8] Kasonga *et al*., conducted a study on the effects of different classes of UFA on osteoblast activity in MC3T3-E1 pre-osteoblasts.[9] DHA, EPA, OA and PLA were shown to modulate osteoblast specific gene expression using the free fatty acid receptor 4-β arrestin 2 signalling pathway thus concluding that this signalling pathway may mediate bone protective effects.[9]

Poulsen *et al*., used MC3T3-E1/4 osteoblast-like cells to determine whether the omega-6 PUFA, AA, stimulates RANKL expression and whether other PUFA such as EPA, DHA and GLA could be used to inhibit RANKL expression in osteoblasts.[174] Secondly, they set out to demonstrate the effects of EPA, DHA and GLA on membrane-bound RANKL expression and OPG secretion in MC3T3-E1/4 cells stimulated with PGE_2 to induce RANKL expression.[174] The results indicated that GLA and AA cyclooxygenase products such as PGE_2 stimulates membrane-bound RANKL expression in MC3T3-E1/4 osteoblast-like cells.[174] Under standard conditions, EPA and DHA had no effect on membrane-bound RANKL expression. However, EPA and DHA inhibited the increase in RANKL expression stimulated by PGE_2.[174] This suggests that EPA and DHA may reduce RANKL-mediated osteoclast formation which could have an impact on bone loss induced by inflammation.[174] In 2013, a study by Casado-Diaz *et al*., examined the effects of AA, EPA and DHA on MSC differentiation into osteoblasts or adipocytes.[175] The study differentiated human MSCs derived from bone marrow in the presence of AA, an omega-6 FA, or EPA and DHA which are omega-3 FA.[175] Markers for osteoblasts or adipocytes were analysed and results showed that AA decreased osteoblast markers and the OPG/RANKL ratio. High concentrations of AA also inhibited mineralisation and stimulated the appearance of adipocytes to a larger extent in comparison to DHA and EPA.[175] Adipogenesis was also increased when the cells were exposed to AA while DHA and EPA did not have an effect.[175] The contradicting results could be due to different types of cell lines used

in the two studies as in Poulsen *et al*., murine osteoblasts cells were used while in the study by Casado-Diaz *et al*., human MSCs were used.

Choi *et al*., led a study to investigate the effects that DHA and ethyl DHA had on MC3T3-E1 osteoblast-like cells.[176] The cells were induced to differentiate into osteoblasts using ascorbic acid and β-glycerophosphate, with or without mouse embryonic fibroblast conditioned medium.[176] DHA showed a higher increase in ALP levels after 2-3 weeks of differentiation and a higher increase in BMP-2 expression compared to ethyl DHA.[176] Coetzee *et al*., studied the effects that AA and DHA had on the differentiation of MC3T3-E1 cells into osteoblasts and trans-differentiation into adipocytes.[177] Results revealed ALP activity was inhibited by long-term exposure of AA while DHA inhibited ALP activity in both long-term and short-term exposure.[177] The study also concluded that AA and DHA did not induce any adipocyte trans-differentiation due to the absence of cytoplasmic triglyceride accumulation.[177] In the study of Choi *et al*., cells were differentiated at intervals of 0, 3, 7, 14 and 21 days before ALP activity was measured while in the study of Coetzee *et al*., cells were differentiated for 48 hours before ALP activity was assessed. This could explain the differences observed in the results between the two studies.

Watkins *et al*., conducted a study on rat foetal calvarial cells, where FA altered RUNX2 gene expression and nodule formation.[8] The effects of LA, AA, conjugated LA, and EPA was determined in rat foetal calvarial cells at 7, 14 and 21 days.[8] Results showed that FA increased the expression of RUNX2 which peaked at 14 days of treatment.[8] LA and AA increased RUNX2 expression to a larger extent but EPA increased RUNX2 expression at 7 and 14 days. Conjugated LA was found to decrease protein expression of RUNX2.[8] Bone nodule formation was determined where separate Alizarin Red S stained aggregates of mineralized bone were counted as nodules.[8] Conjugated LA exerted an inhibitory effect on bone nodules and ALP activity compared to LA.[8] The study concluded that the effects of different FA in rat foetal calvarial cells exert different effects on bone forming cells as LA and AA increased RUNX2 expression however, conjugated LA reduced RUNX2 expression.[8]

2.7.1.2 Osteoclast studies

SFA promoted osteoclast formation in bone marrow macrophages through enhancing the NF-κB pathway and osteoclastic differentiation markers.[178] The effects of palmitate on osteoclast differentiation in RAW264.7 murine macrophages was determined in a study by Drosatos-Tampakaki *et al.,* and found to increase RANKL-mediated osteoclastogenesis, however, the effects of palmitate was counteracted by the presence of OA.[178] Palmitate-mediated osteoclastogenesis was stimulated in the absence of RANKL which could be due to the production of TNF-α, which is also known to stimulate osteoclastogenesis.[178]

The role of PLA, a MUFA, on osteoclast formation was previously determined by van Heerden *et al*.[12] The study investigated the effects of PLA on osteoclast formation in RAW264.7 murine macrophages.[12] It was revealed that PLA exhibited anti-osteoclastogenic properties as it inhibited osteoclast formation induced by RANKL. PLA also affected the expression of osteoclast-specific genes in addition to stimulating apoptosis of mature osteoclasts. In RAW264.7 murine macrophages, PLA was shown to inhibit pERK and pJNK thus supressing the MAPK pathway.[12] Furthermore, activation of NF-κB and MAPK pathways were inhibited which was proposed as a possible mechanism of action for PLA.[12] Previous studies have found that JNK and p38 MAPK are involved in inducing apoptosis while ERK supresses apoptosis.[179-182] Cornish *et al*., explored the effects of SFA, MUFA and PUFA on osteoclastogenesis in RAW264.7 murine macrophages.[183] Results demonstrated that SFA were more potent in inhibiting osteoclast formation in RAW264.7 murine macrophages than omega-3 or -6 PUFA.[183]

Several PUFA have exhibited anti-osteoclastic and anti-osteolytic activities thus exerting a protective effect on bone. In the study by Boeyens *et al*., the effects of omega-3 FA, EPA and DHA, as well as the omega-6 FA, AA and DGLA were examined on RAW264.7 murine macrophages.[184] The PUFA were all found to decrease messenger ribonucleic acid (mRNA) and protein markers of osteoclast differentiation in addition to reducing osteoclastic bone resorption, with AA and DHA being the most effective.[184] Recently, Kasonga *et al*., demonstrated the effects of different classes of UFA on osteoclast activity in RAW264.7 murine macrophages.[9]

DHA, EPA and PLA exerted anti-osteoclastogenic effects through the free fatty acid receptor 4-β arrestin 2 signalling pathway.[9]

Rahman *et al*., examined the effects of DHA and EPA on RANKL-stimulated osteoclast formation in RAW264.7 murine macrophages.[185] The results of this study indicated that DHA is a more potent inhibitor of osteoclastogenesis than EPA. Similarly, DHA significantly reduced NF-κB and p38 MAPK pathway activation thus reducing osteoclast formation and bone resorption.[185] In 2015, Kasonga *et al*., investigated the effects of DHA and AA on osteoclast activity in human CD14+ monocytes isolated from peripheral blood.[186] Data revealed that DHA and AA reduced the potential to differentiate into osteoclasts as well as impaired bone resorption by mature osteoclasts.[186] Osteoclast-specific gene expression was also shown to be downregulated by AA and DHA.[186] This confirms earlier work conducted on RAW264.7 murine macrophages.

Kasonga *et al*., furthermore determined how different classes of UFA activate PPARs and how this may influence osteoclast signalling using human CD14+ monocytes which were differentiated using M-CSF and RANKL.[187] AA, EPA and DHA increased the activity of PPAR-α and PPAR-γ more so than PPAR-β/δ. Conversely, PLA and EPA increased the activity of PPAR-β/δ more than PUFA which suggests that PPAR-β/δ had a higher affinity for MUFA than PUFA in osteoclasts.[187] The study also suggested that this may be a result of the varying concentrations of the UFA used.[187] Fenofibrate, a PPAR-α agonist; Troglitazone, a PPAR-γ agonist; and L-165041, a PPAR-β/δ were shown to significantly reduce osteoclast formation at 10 μM. Troglitazone exerted the most powerful anti-osteoclastogenic effect while fenofibrate exerted the least potent anti-osteoclastogenic effect.[187] The PPAR agonists downregulated RANKL signalling pathway activation and the expression of osteoclast-specific gene expression.[187] The study concluded that PPAR activation inhibits osteoclastogenesis though RANKL signalling modulation.[187]

2.7.2 *In vivo* studies

A number of *in vivo* studies have been conducted on the effects of FA on bone which has shown promising results. Several of these studies focussed on the occurrence/risks of bone fractures in adolescent and elderly populations.[188, 189] Other

studies have reported the effects of omega-3 and -6 PUFA in animal and human models.[173, 188, 190, 191]

2.7.2.1 Animal studies

In a study by Lanham *et al*., high fat/high calorie diets in maternal mice during gestation were found to have an effect on their offspring's bone structure by increasing the bone marrow adiposity in the femur compared to the control diet.[192] The high fat diet consisted of 18% animal lard which contains mostly SFA and some PUFA as well as MUFA. Mothers fed on the high fat diet also showed altered in utero offspring developmental organisation as their female offspring presented with an altered trabecular structure. Thus, in utero nutrition can affect offspring health thus having consequences later on in life.[192] In a study by Drosatos-Tampakaki *et al*., OA enhanced triglyceride formation which prevented osteoclastogenesis by PA.[178] Mice fed on PA-enriched high-fat diets showed larger reductions in bone mass and structure than mice fed OA-enriched high-fat diets.[178] SFA enhanced RANKL-mediated osteoclastogenesis through upregulating TNF-α and RANK.

A study led by Mousavi *et al*., found that the consumption of extra-virgin olive oil, which is high in OA, during mice gestation and lactation caused OPG and the OPG/RANKL ratio mRNA to be upregulated in the offspring's bone thus promoting osteoblastogenesis.[193] However, offspring born to mice who were fed 45% extra-virgin olive oil compared to 16% extra-virgin olive oil showed an increase in PPAR-γ gene expression and a reduction in OPG and OPG/RANKL mRNA expression thus promoting adipogenesis and osteoclastogenesis.[193] The study concluded that the type and the amount of extra-virgin olive oil fed to maternal mice affected bone remodelling markers in adolescent offspring.[193] Mousavi *et al*., also explored the effects of extra-virgin olive oil in maternal mice diets and found that the consumption of this increased osteoblastic gene expression in adolescent offspring when compared to soybean oil.[193] However, high amounts of both the oils caused a decrease in osteoblast gene expression and an increase in adipogenic gene expression in adolescent offspring.[193]

In 2010, Halade *et al*., set out to establish a model for high fat diet-induced obesity with the aim of associating this condition to osteopenia and osteoporosis.[173] They fed

10% corn oil to aging female mice, as a high omega-6 dietary fat source is commonly observed in Western diets.[173] They concluded that the corn oil fed to these insulin-resistant and obese female mice, decreased the BMD which was also accompanied with increased PPAR-γ expression, increased bone marrow adiposity and pro-inflammatory cytokines in the bone marrow.[173] Thus mimicking human chronic conditions experienced during obesity and post-menopausal bone loss. This model could therefore be useful for future studies on obesity, age-related bone loss, and dietary fatty acids.

Chen *et al*., conducted an investigation on the effects of endogenous omega-3 PUFA on bone marrow adiposity under conditions of osteoporosis using ovariectomised (OVX) FAT1 transgenic mice as their osteoporosis model.[194] These mice convert omega-6 PUFA to omega-3 PUFA endogenously at a fixed ratio and overexpress the FAT1 gene.[194] Results showed that omega-3 PUFA inhibited the accumulation of bone marrow adiposity which prevents bone loss induced by ovariectomy.[194] Transgenic mice displayed a significantly lower PPAR-γ expression compared to wild-type mice, in addition to a higher RUNX2 gene expression in the bone marrow.[194] The study concluded that the endogenous conversion of omega-6 to omega-3 PUFA attenuates bone marrow adipogenesis which is associated with an increase in RUNX2 expression and a decrease in PPAR-γ expression.[194]

OVX rats fed EPA enriched diets inhibited oestrogen-deficiency induced bone weight loss and strength. Thus, omega-3 PUFA may play a role in maintaining bone health.[8] On the other hand, mice fed diets enriched with PA displayed a larger reduction in bone mass and structure than mice fed OA enriched diets.[14] In a study by Song *et al*., ALA, an omega-3 PUFA, was found to inhibit RANKL-mediated osteoclastogenesis in OVX mice through repressing RANKL-induced markers of osteoclasts and the phosphorylation of ERK.[14, 191]

A study by Anez-Bustillos *et al*., concluded that omega-3 PUFA had no beneficial effects on bone in healthy young mice and suggested the beneficial effects of omega-3 PUFA may be only become evident in diseased states.[190] The study was limited as it lacked single housing for mice as well as adequate food intake control for each mouse. The activity of the mice were also uncontrolled which can impact bone health.[190]

The effects of omega-3 and -6 FA such as EPA, DHA, GLA and a mixture of all three (MIX) were analysed in OVX rats in a study conducted by Poulsen *et al*.[195] Results indicated that DHA improved bone mineral loss induced through ovariectomy. MIX and EPA did not exhibit any bone protective effect against bone loss in the study, however, post-OVX bone mineral loss was exacerbated by GLA.[195]

Li *et al*., conducted a study using omega-3 FA deficient rats to determine if preformed dietary docosapentaenoic acid (DPA) could replace DHA for normal bone growth.[196] The pups were fed artificial rat milk containing ALA in combination with either DHA, DPA or a combination of both (DHA/DPA). At adulthood, the rats were euthanized and their BMD and bone mineral content (BMC) analysed.[196] Results indicated that when there was a lack of omega-3 FA in the diet, omega-6 FA replaces omega-3 FA in the bone but does not allow for normal bone growth. Rats fed DPA contained higher DPA content and presented with the lowest BMC and BMD.[196]

2.7.2.2 Human studies

A study conducted by Corwin *et al*., analysed the effects of SFA in American men and women using data collected from the National Health and Nutrition Examination Survey III.[197] This survey set out to assess whether there is an association between certain FA, total dietary fat and BMD in the proximal femur of the hip region.[197] This site is important as it is a vulnerable site for fractures in the elderly.[197] Results indicated that SFA intake was negatively associated with bone density, especially in men over 50 years of age.[197] High dietary SFA not only increased the risk for cardiovascular disease, but also may increase the risk of bone disease.[197]

There have been a number of studies conducted on human populations on the effects of FA and bone but some have shown contradictory results. One study concluded that a change in the omega-6/omega-3 ratio from 10:1 to 2:1 had no effect on bone resorption or formation markers.[198] However, another study concluded that a decrease in omega-6/omega-3 ratio resulted in a decrease in bone resorption markers and exerted anti-inflammatory effects.[199] The decreased ratio of omega-6/omega-3 PUFA can reduce inflammation and thus the severity of skeletal conditions such as rheumatoid arthritis and osteoporosis.[153]

Plant sources of omega-3 PUFA may exert a protective effect on bone metabolism. Griel *et al*., conducted a controlled feeding study involving middle-aged men and found that the incorporation of walnuts and flaxseed in the diet caused a decrease in the omega-6/omega-3 PUFA ratio which exerts an anti-inflammatory effect and reduces resorption markers in bone.[199]

Rajaram *et al*., evaluated bone turnover biomarkers after dietary omega-6/omega-3 ratio alteration using plant or marine supplementation in healthy adults.[198] Twenty-four healthy adults took part in the study where participants were given one of four diets: a control diet, an EPA/DHA diet, an ALA diet or a combination diet.[198] Results suggest the diets did not affect any bone resorption or formation markers, however, the study also stated that the lack of favourable results could be due to a low bone turnover rate in young, healthy adults.[198] The small sample size of the study could have influences the outcome of the study.

Weiss *et al*., described the association between omega-6/omega-3 ratio in BMD in 1 532 men and women from data collected from 1988-1992.[200] Results indicate a significant inverse relationship between the LA/ALA ratio and hip BMD.[200] The study concluded that a higher ratio of omega-6/omega-3 is associated with a lower hip BMD in men and women.[200]

A recent study by Vinding *et al*., examined the effects of omega-3 PUFA supplementation during pregnancy on offspring.[201] The study involved 736 pregnant women who were supplemented with either fish oil or olive oil as a control from the 24th week of pregnancy up until one week after birth.[201] The offspring were found to have a higher body mass index (BMI) from 0 to 6 years of age with no increase in obesity risk.[201] The offspring given fish oil were also found to have increased bone, lean and fat mass suggesting their overall growth was stimulated.[201]

Some studies did not find any beneficial effects of PUFA on the risk of hip fractures in human populations while other studies attributed hip fracture risk to other lifestyle and dietary factors such as green tea consumption and the number of children born to women. Virtanen *et al*., conducted a study in investigating the effects of fish consumption along with EPA and DHA consumption on the BMD and risk of hip

fractures in the elderly.[202] The study involved 5 045 men and women who were 65 years of age or older.[202] Results suggested that fish consumption and estimated EPA and DHA consumption did not significantly lower the risk of hip fractures.[202] A high intake of tuna and other fish was found to have a small association with lower BMD at the femoral neck and there was no apparent dose-dependent relationship.[202] A study led by Maraki *et al*., demonstrated that increased BMD in Japanese women could be due to the consumption of green tea which contains flavonoids that have been shown to induce osteoclast apoptosis.[203] The study involved 632 women who were 60 years or older.[203] The women who drank alcohol and green tea, and were physically active, had a higher BMD than the women who smoked.[203] The results of this study could suggest that the consumption of fish or PUFA may not be the only factor which determines bone health in populations known for a high PUFA intake.[203]

Farina *et al*., demonstrated the relationship between hip BMD, and dietary PUFA and fish intake in adults taking part in the Framingham Osteoporosis Study.[204] The study found that high intake of fish was associated with a lower rate of bone loss at the four year follow up in the femoral neck in men and women, depending on the type of fish consumed.[204] AA exerted a protective effect in women with a high intake of EPA and DHA but not in those with low intakes.[204] The effects of EPA, DHA and AA were overall protective in men, however, AA was detrimental to men with a low intake of EPA and DHA.[204] Higher LA intake was also associated with higher rates of bone loss in women and not in men.[204] The study concluded that AA may exert protective effects on bone depending on EPA and DHA intake.[204]

In 2016, a short-term study conducted by Fonolla-Joya *et al*., aimed to evaluate the effects of dairy products enriched with PUFA on bone metabolism in 117 healthy postmenopausal women.[205] The women were given low-lactose skimmed milk enriched with EPA and DHA, or OA. UFA had no effect on bone turnover markers or OPG in the treatment groups, however, RANKL was reduced which may indicate a reduction in bone resorption.[205] A high bone turnover may results in an increased fracture risk in postmenopausal women thus a reduction in bone resorption could result in a reduced fracture risk.[205]

A secondary analysis of cross-sectional data from 6 cohorts of postmenopausal women was performed by Harris *et al.,* in 2015.[206] Mean intake of dietary PUFA were

assessed and results showed that ALA and LA was significantly inversely associated with total body and lumbar spine BMD. AA had a positive association to BMD in the trochanter.[206] In the hormone therapy group, which consisted of women supplemented with hormone therapy, ALA and LA together had a positive association to total body BMD while ALA alone had an inverse relationship to total body and spine BMD.[206] This study demonstrates that hormone therapy may modulate the effects of PUFA on bone.[206]

To conclude, the beneficial effects of PUFA on human bone health have been identified in some studies[204, 207-211] but not in others.[202, 203, 212] There are several challenges between nutrition and bone health which have been commonly outlined and these include [1] very few studies were reported that look at the cumulative effects of diets throughout life, [2] deleterious bone outcomes usually only become evident later on in life, [3] single dietary nutrients make a small contribution to dietary composition thus a whole-diet approach seems to be a superior method to analyse the effects of diets on bone health, and [4] diets depend on the socioeconomic status and lifestyle decisions of an individual as factors such smoking and physical inactivity affects bone health.[170] Bone health is likely more influenced by the effects of a mixed type of nutrients in a diet rather than a single nutrient.[14]

Chapter 3

Materials and methods

3.1 Logistics

All experiments were conducted in the laboratories of Department of Physiology and the Centre for Neuroendocrinology at the University of Pretoria. Photomicrographs were taken at the Department of Physiology at the University of Pretoria.

3.2 Ethical consent

Ethical consent was obtained from the Faculty of Health Sciences Research Ethics Committee at the University of Pretoria. (Reference number 386/2018, Appendix A)

3.3 General laboratory procedures

3.3.1 Materials and reagents

PLA was bought from Cayman Chemical Company (Ann Arbor, MI, USA). Resazurin, trypan blue, Triton X-100, 1% antibiotic solution (fungizone, streptomycin and penicillin), goat anti-rabbit IgG antibody horseradish peroxidase (HRP) conjugate and all other chemicals of research grade was purchased from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagles Medium (DMEM) (Cat #11594486) and Trypsin-ethylenediaminetetraacetic acid (EDTA) (Cat #10779413) were purchased from GIBCO (Invitrogen Corp, Carlsbad, CA, USA) or from Biowest (Cat #X0930-100) (MO, USA), and heat inactivated foetal bovine serum (FBS) was bought from Capricorn Scientific (Cat #FBS-GI-12A) (Ebsdorfergrund, Germany). Consumable plastic ware such as culture flasks, culture plates, pipette tips, syringes, etc. were purchased from Lasec (Midrand, GP, SA). Clarity Western ECL Substrate was bought from Bio-RAD (Hercules, CA, USA). M-MuLV reverse transcriptase was purchased from New England Biolabs (Ipswich, MA, USA). The bicinchonic acid (BCA) protein assay kit was purchased from Thermo-Fischer Scientific (Rockford, IL, USA). Primary antibodies against p38 MAPK was supplied by Abcam (Cambridge, MA, USA); antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and pp38

MAPK was supplied by Sigma Aldrich (St. Louis, MO, USA); antibodies against JNK and ERK was supplied by Signalway Antibody (College Park, MD, USA); and antibodies against pJNK and pERK was supplied by Research and Diagnostic systems (R & D systems, Minneapolis, MN, USA). Primers for ALP, RUNX, OCN Osx, OPG, GAPDH and RANKL were synthesized and purchased from Inqaba Biotec (Pretoria, GP, SA).

3.3.2 Preparation of buffers and solutions

Table 1. Preparation and storage of buffers and solutions used in this study.

Compound	Preparation and storage
Buffers: 1. Phosphate buffered saline (PBS)	For 10X stock solution: 80 g NaCl, 2 g KCl, 2 g KH_2PO_4 and 10,5 g Na_2HPO_4 in 1 L double distilled water (ddH_2O). pH adjusted to 7.4. Working solution: stock solution diluted 10 times in ddH_2O and autoclaved. Aliquots stored at 4˚C in the dark.
2. Trypsin-EDTA working solution	50 mL Trypsin-EDTA prepared in 450 mL autoclaved sterile PBS. Aliquots stored at -20˚C in the dark.
3. Freeze medium	40% FBS, 50% serum free DMEM and 10% dimethyl sulfoxide (DMSO).
4. Trypan blue dye, 0.5%	0.5 g prepared in 100 mL PBS.
5. Resazurin solution, 0.02% (w/v)	0.02 g diluted in 100 mL ddH_2O.
6. Osteogenic media (OM): β-glycerophosphate	For 10 mM solution: 0.017 g β-glycerophosphate prepared in 8 mL complete DMEM*.
Ascorbic acid	50 mM prepared in ddH_2O. Aliquots stored at -80˚C in the dark, diluted to 50 µM in complete DMEM*.
Dexamethasone	1 mM prepared in 100% ethanol. Aliquots stored at -80˚C in the dark, diluted to 1 µM in complete DMEM*.

7. ALP assay buffer	5 mM 4-nitrophenyl phosphate disodium, 0.5 mM $MgCl_2$, 0.1% Triton X-100 in 50 mM Tris-HCl with the pH adjusted to 9.5.
8. Alizarin Red S staining solution, 2% (w/v)	0.2 g Alizarin Red S prepared in 10 mL ddH_2O.
9. Lysis buffer: RIPA buffer	50 mM Tris, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA and 50 mM NaF with the pH adjusted to 7.4.
10. Loading buffer (6X)	0.5 M Tris [pH 6.8], 10% SDS, 30% glycerol, 0.03% bromophenol blue and 6% β-mercaptoethanol.
11. Tris-glycine transfer buffer	For 10X stock solution: 144 g glycine and 30.3 g Tris, filled to 1 L with ddH_2O. Stored at room temperature. For 1X solution: 100 mL 10X Tris-glycine transfer buffer, 200 mL methanol and 700 mL ddH_2O.
12. Oil Red O solution	For 0.5% (w/v) stock solution: 0.25 g Oil Red O diluted in 50 mL isopropanol. Stored for up to 3 months at room temperature. For a 60% (v/v) working solution: 4.2 mL Oil Red O stock solution and 2.8 mL ddH_2O. Incubate solution for 10 minutes and then filter the solution.

*Complete DMEM - DMEM supplemented with 10% FBS

Table 2. Preparation and storage of primers and antibodies used in this study.

Compound	Preparation and storage
1. Primers: GAPDH ALP RUNX2 Osx OCN OPG RANKL	All primers were reconstituted to 100 µM in nuclease free water. For 10 µM working solution: 10 µL of forward and 10 µL reverse primer prepared in 80 µL nuclease free water. Aliquots stored at -20˚C in the dark. (Primer sequences can be found in Table 3)
2. Antibodies: GAPDH ERK pERK	1 mg/mL in PBS. Aliquots stored at -20˚C in the dark.

3.3.3 Cell culturing and maintenance

ASCs were isolated from human abdominal subcutaneous adipose tissue from donors undergoing liposuction as previously described, and preserved in liquid nitrogen.[213] These cells were made available (by the Institute of Cellular and Molecular Medicine, University of Pretoria) for use in this study. ASCs were maintained in complete ASC media (DMEM supplemented with 10% FBS and 1% antibiotic solution containing 0.25 µg/mL fungizone, 100 µg/mL streptomycin and 100 ug/mL penicillin).[6]

The MG-63 osteosarcoma cell line (MG63-C) was purchased from Cellonex (South Africa). MG-63 cells were maintained in complete DMEM supplemented with 10% FBS.[214] MG-63 osteosarcoma cells were chosen for this study as these cells have previously successfully been used as an *in vitro* model for osteoblastogensis.[215] The cell line has a doubling time of 38 hours and can be cultured indefinitely.[215, 216]

All cells were cultured under sterile conditions at 5% CO_2 and 37˚C in a humidified incubator. Cells were sub-cultured at 80% confluency in T-75 flasks by removing the medium, rinsing the cells twice with sterile PBS (Table 1 #1) and adding 5 mL Trypsin-EDTA (Table 1 #2) for 7 minutes at 37˚C in a humidified incubator.[6] Fresh medium was added, the cells were aspirated and placed into a 15 mL tube, and were centrifuged. The supernatant was removed and fresh media was used to re-suspend

the cells. The cells were aspirated and 500 000 cells were placed into a new culture flask. Medium was replaced 1-2 times per week.[9] To freeze cell stock away, cells were frozen in cryovials at a density of $1x10^6$ cells per mL in freeze medium (Table 1 #3) and placed into long-term storage in liquid nitrogen when not in use, or in -80˚C freezer for short-term storage. The cell count, passage and dates were recorded. ASCs were only used in experiments up until a passage of 10, while MG-63 cells were only used in experiments up until a passage of 30. All cell culture work was performed in a sterile laminar flow cabinet to guarantee there was no contamination.

3.3.4 Palmitoleic acid and vehicle preparation (Stock solution preparation and storage)

Stock concentrations of 10 mM PLA were prepared in ethanol. Aliquots were stored at -80°C in the dark until required. The stock solutions were freshly diluted to the required concentrations ranging from 20-100 µM in complete DMEM. The highest concentration of ethanol did not exceed 0.1% during the experiments and was used as the vehicle control (VC).[186]

3.3.5 Trypan blue exclusion and cell counting for experiments

Cell counts were obtained using Trypan blue dye exclusion and a haemocytometer. Cells were trypsinized, centrifuged and re-suspended in DMEM. Twenty microliters cell suspension was added to 80 µL PBS. A 1:1 working solution was prepared using 20 µL of Trypan blue dye (Table 1 #4) and 20 µL of the cell solution.[217] Thereafter, 20 µL was pipetted onto the haemocytometer under a coverslip. The viable cells were counted in four quadrants using an Olympus CKX53 microscope equipped with an SC30 camera (Olympus, Tokyo, Japan).[217] Non-viable cells stained blue and were not counted. The total number of viable cells over the four quadrants was averaged and multiplied by 10^5 to obtain the number of viable cells per mL.[217]

3.4 Methods for experiments

3.4.1 Resazurin assay for the detection of cell viability

A cell viability assay was used to assess the number of viable cells after the addition of a treatment or compound. Resazurin is specifically used to measure the cytotoxicity

or proliferation of a compound.[218] ASCs and MG-63 osteosarcoma cells were seeded at 5 000 cells per cm^2 and 10 000 cells per cm^2 into a sterile 96-well plate in complete ASC media and complete DMEM (VC), respectively, and incubated for 24 hours to allow attachment. A control was used which contained 0.1% Triton X-100. Triton X-100 permeabilized the cell membranes and therefore inhibited the metabolic activity of the cells.[219] Osteogenic media or varying concentrations of PLA (20, 40, 60, 80 and 100 µM) were added to the cells and incubated for a further 24 hours. At the end of the culture period, a 10% resazurin working solution made up of 0.02% resazurin solution (Table 1 #5) and complete DMEM was added to each well in order to assess the metabolic activity of the cells. The plates were then incubated for 1-4 hours at 37ºC in the dark until the solution turned light purple/violet.[219]

The active ingredient resazurin is water-soluble and non-toxic. The solution is dark blue and is reduced to a fluorescent light purple/violet colour known as resorufin as it is an electron acceptor.[219] Once the solution turned purple, absorbance was measured using an Epoch Micro-plate spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA) at a wavelength of 570 nm and a reference wavelength of 600 nm.[12, 219] Cell viability was calculated according to the Bio-RAD protocol for the alamarBlue spectrophotometry. Three independent experiments were conducted and each sample was seeded in triplicate.

3.4.2 Osteoblast differentiation

For osteogenic differentiation to occur, complete DMEM supplemented with OM containing β-glycerophosphate, ascorbic acid and dexamethasone (Table 1 #6) is required for at least 21 days. Dexamethasone prevents apoptosis from occurring and also enhances MSC proliferation.[220-222] It further enhances osteoblast differentiation by modulating RUNX2 phosphorylation.[223] β-glycerophosphate serves as a source of phosphate needed for hydroxyapatite formation as well as for the phosphorylation of ERK1/2, and ascorbic acid enhances osteogenic differentiation through the secretion of COL1.[223]

3.4.3 Alkaline phosphatase (ALP) activity assay as an early marker for the detection of osteoblast differentiation

Alkaline phosphatase is an early marker for osteoblast differentiation as it is an enzyme which is released during bone formation and therefore is a by-product of osteoblastogenesis.[224] ASCs and MG-63 cells were seeded at 5 000 cells per cm^2 and 10 000 cells per cm^2 into a sterile 48-well plate in complete ASC media and complete DMEM, respectively, and incubated for 24 hours to allow attachment. Cells were differentiated in the presence of OM and PLA (20, 40, 60, 80 and 100 µM) which was replaced every 2-3 days. ALP activity assay was used to indicate the presence of osteoblast cells after 21 days of differentiation.[225] Three independent experiments were conducted and each sample was seeded in duplicate.

At the end of culture, cells were fixed with 4% formaldehyde in PBS for 15 minutes. ALP was assayed using a colorimetric assay where *p*-nitrophenylphosphate is converted into *p*-nitrophenol. The fixed cells were incubated with ALP assay buffer (Table 1 #7) for 60 minutes at 37˚C.[177] Subsequently, 100 µL of the ALP assay buffer was transferred to a new 96-well plate and absorbance was read at 405 nm wavelength using an Epoch Micro-plate Spectrophotometer (Biotek Instruments Inc., Winooski, VT, USA).[177]

3.4.4 Alizarin Red S staining for the detection of calcium in the mineral matrix

Alizarin Red S staining is used to stain calcium secreted by tissues such as osteogenic lineage cells.[226] ASCs and MG-63 osteosarcoma cells were seeded at 5 000 cells per cm^2 and 10 000 cells per cm^2 into a sterile 48-well plate in complete ASC media and complete DMEM, respectively, and incubated for 24 hours to allow attachment. Cells were differentiated in OM in the presence of PLA (20, 40, 60, 80 and 100 µM) for 21 days. Media and all factors were replaced every 2-3 days.

At the end of culture, Alizarin Red S staining was performed whereby the cells were fixed with 3.7% formaldehyde for 1 hour. The cells were subsequently washed with PBS at a pH of 4.2 for 5 minutes and then stained in a 2% (w/v) solution of Alizarin Red S (Merck, Germany) (Table 1 #8) for 10 minutes.[6] Thereafter, the stained cells were rinsed with ddH_2O and visualised by light microscopy. Photomicrographs were

taken using an Olympus CKX53 microscope equipped with an SC30 camera (Olympus, Tokyo, Japan).[6, 225]

Thereafter, for quantification, Alizarin Red S was eluted using 200 µL 10% acetic acid and neutralized with 200 µL 10% ammonium hydroxide where it was transferred to a new 96-well plate. The absorbency was read at 405 nm using an Epoch Micro-plate Spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA).[226] Three independent experiments were conducted and each sample was seeded in duplicate.

3.4.5 Quantitative-Polymerase Chain Reaction for the detection of osteoblast specific genes

Quantitative polymerase chain reaction, a method used to analyse mRNA levels was used to study the effects of PLA on osteoblast specific markers. ASCs and MG-63 osteosarcoma cells were seeded at 5 000 cells per cm^2 and 10 000 cells per cm^2 into a sterile 48-well plate in complete ASC media and complete DMEM, respectively, and incubated for 24 hours to allow attachment. The cells were then differentiated in the presence of OM and PLA (100 µM) which was replaced every 2-3 days.

Osteoblasts were analysed for early bone specific gene expression markers for up to 14 days of differentiation. The data was analysed using the $2^{-\Delta\Delta CT}$ method after results were normalized using GAPDH, as PLA did not affect GAPDH expression.[12] Genes for early bone biomarkers were analysed after 7 and 14 days of differentiation. These markers include ALP, RUNX2, Osx, OCN, OPG and RANKL. Genes for late bone biomarkers such as ALP, OCN, BSP and PPAR-γ could not be analysed at 21 days of differentiation as planned due to the difficulty in extracting mRNA from the mineralised matrix.

Messenger ribonucleic acid was extracted using 300 µL TRI reagent (Thermo-Fischer Scientific, USA).[227] The samples were left at room temperature for 5 minutes to allow dissociation of the nucleoproteins, thereafter, 60 µL chloroform was added and the samples were vortexed and incubated at room temperature for 2-3 minutes.[227] The samples were centrifuged at 12 000 xg for 15 minutes at 2-8˚C where the mixture separated into a red phenolchloroform layer, an interphase layer and an aqueous layer. The RNA was present in the aqueous layer which was transferred and

precipitated by adding 0.5 mL of isopropyl alcohol per 1 mL trizol reagent. The RNA was subsequently incubated for 10 minutes at 15-30˚C and centrifuged at 12 000 xg for 10 minutes at 4˚C where a gel pellet formed. Thereafter, the supernatant was removed completely and the pellet was washed twice using 300 µL of 75% ethanol. The ethanol was removed and the pellet was then air-dried and dissolved in 10 µL DNAse and RNAse free polymerase chain reaction (PCR)-grade water. RNA was quantified using a NanoDrop 1000 Spectrophotometer (ThermoFischer Scientific, MA, USA), where a 260/280 ratio of 2 was accepted as pure RNA.

Complementary DNA (cDNA) was reverse transcribed from RNA using M-MuLV reverse transcriptase and incubated in the oven at 42˚C for 1 hour.[227] Thereafter, the enzyme was inactivated at 90˚C and the DNA was stored at -20˚C until needed. The primers used in this study (Table 2 #1) are osteoblast-specific genes and were synthesized by Inqaba Biotec (Pretoria, South Africa) using the sequences given in Table 3. The cDNA was added to forward and reverse primers for the osteoblast-specific genes.[225]

Luna Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) along with GAPDH and gene primers, were used for qPCR.[12, 228] The samples were centrifuged, placed in a Lightcycler nano (Roche, Switzerland) and run on a 3-step cycling using the cycles given in Table 4. Three independent experiments were conducted and each sample was seeded in duplicate.

Table 3: Primers which were used for the qPCR.

	Forward primer sequence (5' – 3')	**Reverse primer sequence (5' – 3')**
GAPDH	GAAAGCCTGCCGGTGACTAA	GCCCAATACGACCAAATCAGAG
Alkaline phosphatase	CTATCCTGGCTCCGTGCTCC	GTTAACTGATGTTCCAATCCTGCG
RUNX2	TGCTTCATTCGCCTCACAAA	GCCTTAAATGACTCTGTTGGTC
Osterix	GAGTGGAACAGGAGTGGAGC	GATGGAGAGAGCTGGGGGAA
Osteocalcin	CCTCTCCGGGATGGTCTGTG	CCCGTGCAAAATGCCCATC
OPG	TGGAATAGATGTTACCCTGTGTGA	TGCGTTTACTTTGGTGCCAG
RANKL	ATGTTCGTGGCCCTCCTG	GGATCCATCTGCGCTCTGAAA

Table 4: The cycles and their duration used in the Lightcycler nano for qPCR.

Cycles	Temperature	Duration	Step
1	95˚C	1 minute	Polymerase activation
40-45	95˚C	15 seconds	Denaturing
	60˚C	30 seconds	Extension
1	60-95˚C	various	Melt curve

3.4.6 Western blotting for the detection of mitogen-activated protein kinase (MAPK) protein expression

Western blotting is a method used to examine specific proteins such as those involved in the MAPK pathway through gel electrophoresis.[12] MG-63 osteosarcoma cells were trypsinized and seeded at 104 000 cells per cm^2 into a sterile 6-well plate in complete DMEM and incubated for 24 hours to allow attachment. Cells were treated for 3, 6 and 24 hours in OM and in the presence of PLA (100 µM). Cells were then scraped in ice cold Tris-buffered saline (TBS) and the samples were placed on ice. The cells were lysed in ice-cold lysis buffer (Table 1 #9) supplemented with protease, phosphatase inhibitors and phenylmethane sulfonyl fluoride (PMSF) for 30 minutes on ice.[229] The samples were subsequently vortexed every 10 minutes. Thereafter, the lysed cells were centrifuged at 15 000 xg for 10 minutes at 4˚C to extract total cellular proteins. The supernatant was stored at -80˚C in the dark until needed.[229]

The extracted proteins were quantified using a BCA protein assay kit.[12] Thereafter, loading buffer (Table 1 #10) was added and the samples were denatured at 60-90˚C for 10 minutes. Equal amounts of proteins were then fixed on a 12% polyacrylamide gel. Proteins were electrotransferred onto nitrocellulose membranes using a Tris-glycine transfer buffer (Table 1 #11). TBS, pH 7.4 with 0.05% Tween-20 (TBS-T) solution containing 5% bovine serum albumin (BSA) was used to block the membranes for one hour.[229] The membranes were probed with specific primary antibodies and GAPDH (housekeeping antibody) (Table 2 #2) at 4˚C overnight. The membranes were washed 3 times at 10 minute intervals using 5 mL TBS-T and then incubated with goat anti-rabbit IgG antibody HRP conjugate (1:20 000) for one hour. The blots were washed again as described previously and thereafter, developed using the Bio-RAD Clarity Western ECL substrate and a Chemidoc MP (Bio-RAD, Hercules,

CA, USA). The images were analysed making use of ImageJ software and densitometric data for band intensities was generated.[12, 230] Three single independent experiments were conducted.

3.4.7 Oil Red O staining for the detection of adipocytes

Oil Red O is a dye used to stain neutral triglycerides such as those found in adipocytes. An Oil Red O staining procedure was performed to ensure that cells did differentiate into osteoblasts and not adipocytes. A 0.5% Oil Red O stock solution (w/v) was prepared in isopropanol followed by a 60% working solution (Table 1 #12).[6]

ASCs and MG-63 osteosarcoma cells were seeded at 5 000 cells per cm^2 and 10 000 cells per cm^2 into a sterile 48-well plate in complete ASC media and complete DMEM, respectively, and incubated for 24 hours to allow attachment. Cells were then differentiated in the presence of OM and PLA (20, 40, 60, 80 and 100 µM) which was replaced every 2-3 days. Differentiated cells were analysed for the presence of mature adipocytes after 21 days of differentiation.

At the end of the culture period, cells were fixed in 3.7% formalin prepared in PBS and incubated for 30-60 minutes at room temperature. The formalin was discarded and the cells were rinsed with ddH_2O. One hundred microliters Oil Red O staining solution was added to each well and incubated at room temperature for 5 minutes.[6] The staining solution was rinsed off until clear and thereafter, haematoxylin was added as a counterstain for 1 minute at room temperature. Subsequently, the haematoxylin was rinsed off and the cells were left to dry completely.[231] Cells were then visualised by light microscopy and photomicrographs were taken using Olympus CKX53 microscope equipped with an SC30 camera (Olympus, Tokyo, Japan).

For quantification, the Oil Red O stained lipid droplets within adipocytes were eluted by adding 500 µL of 100% isopropanol to each well for 10 minutes at room temperature.[232] One hundred microliters was then transferred from each well into a new 96-well plate and absorbance was read at 500 nm using an Epoch Micro-plate Spectrophotometer (BioTek Instruments Inc., Winooski, VT, USA).[6, 231] Three independent experiments were conducted and each sample was seeded in duplicate.

3.5 Sample size and data analysis

Three independent experiments were conducted in triplicate unless otherwise stated, and exposed cells were compared to the VC+OM. Results were displayed as the mean ± the standard deviations. Statistical analysis was done using appropriate one or two-way analysis of variance (ANOVA), followed by Bonferroni's post test to determine the effects of PLA on cell viability, alkaline phosphatase activity, calcium mineralisation, adipogenesis, gene and protein expression, in MG-63 osteosarcoma cells and ASCs. Linear contrasts have been evaluated between different concentrations. P-values<0.05 were considered significant. Data analysis methods were established in a consultation with Prof PJ Becker from the Biostatistics Unit in the Faculty of Health Sciences Research Office, University of Pretoria. GraphPad Prism version 5.00 software, (GraphPad Software Inc., California, USA) was used to obtain the statistical analysis of the data.

Chapter 4

Results

4.1 Cell viability: Resazurin assay

The effects of PLA on cell viability in undifferentiated human ASCs and MG-63 osteosarcoma cells was investigated using a resazurin assay. Cells were treated using various concentrations of PLA determined in previous studies for 24 hours and thereafter, cell viability was determined.[12] A resazurin assay contains resazurin, a blue dye which becomes reduced to a purple state. The intensity of the reduced state is proportional to the amount of living cells and thus can be used as an indicator of cell viability.[219]

4.1.1 The effects of palmitoleic acid on cell viability in undifferentiated adipose-derived stromal cells

ASCs exposed to OM (containing differentiation factors) and 20-100 µM PLA for 24 hours showed no significant effect on cell viability when compared to cells grown in media in the absence of differentiation factors (VC) as seen in Figure 4.1. Triton X-100 was included to observe the effects when cells are not metabolically active as this permeabilizes the cell membrane and harms the cell resulting in a decreased cell viability. Triton X-100 significantly reduced ($p<0.001$) the cell viability of the ASCs compared to the VC.

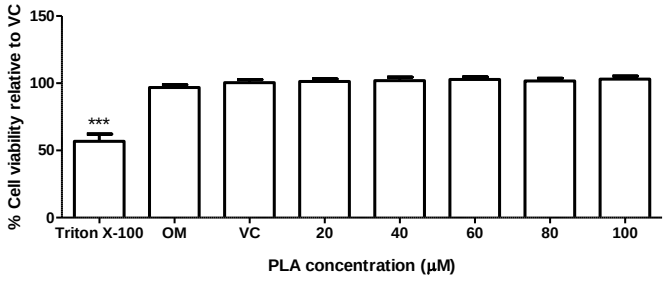

Figure 4.1. The effects of PLA on cell viability in ASCs after 24 hours of exposure.

ASCs were seeded at 5 000 cells per cm^2 in a 96-well plate and were allowed to attach. Cells were exposed to a range of PLA concentrations (20, 40, 60, 80, 100 µM) and OM for 24 hours. Cell viability was expressed relative to the vehicle control (VC). Data are representative of three independent biological repeats which were performed in triplicate. ***$p<0.001$ vs VC.

4.1.2 The effects of palmitoleic acid on cell viability in undifferentiated MG-63 osteosarcoma cells

MG-63 cells exposed to OM and 20-100 µM PLA for 24 hours showed no significant decreases in cell viability when compared to cells grown in the absence of differentiation factors (VC) as shown in Figure 4.2. Triton X-100 was included to observe the effects when cells are not metabolically active as this permeabilizes the cell membrane and harms the cell resulting in a decreased cell viability. Triton X-100 significantly reduced ($p<0.001$) cell viability of the MG-63 cells compared to the VC.

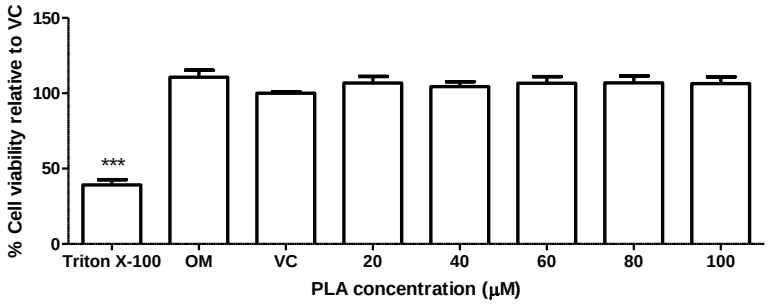

Figure 4.2. The effects of PLA on cell viability in MG-63 cells after 24 hours of exposure.
MG-63 osteosarcoma cells were seeded at 10 000 cells per cm^2 in a 96-well plate and were allowed to attach. Cells were exposed to a range of PLA concentrations (20, 40, 60, 80, 100 µM) and OM for 24 hours. Cell viability was expressed relative to the VC. Data are representative of three independent biological repeats which were performed in triplicate. ***$p<0.001$ vs VC.

4.2 Alkaline phosphatase activity assay

The effects of PLA on ALP activity was assessed in ASCs and MG-63 osteosarcoma cells using ALP activity assay. Cells were differentiated and treated with various concentrations of PLA for up to 21 days. Thereafter, ALP activity was measured and represented graphically. ALP is an enzyme which increases when osteoblasts are active.

4.2.1 The effects of palmitoleic acid on alkaline phosphatase activity in differentiated adipose-derived stromal cells

ASCs were exposed to 20-100 μM PLA for up to 21 days in the presence of OM. Overall, a higher ALP activity was detected at 7 days compared to 14 and 21 days (Figure 4.3). ALP activity peaked at 60 μM PLA, this was however not significant compared to the VC in the presence of osteogenic factors (VC+OM). The ALP activity remained constant over all PLA concentrations for the 14 and 21 days of exposure.

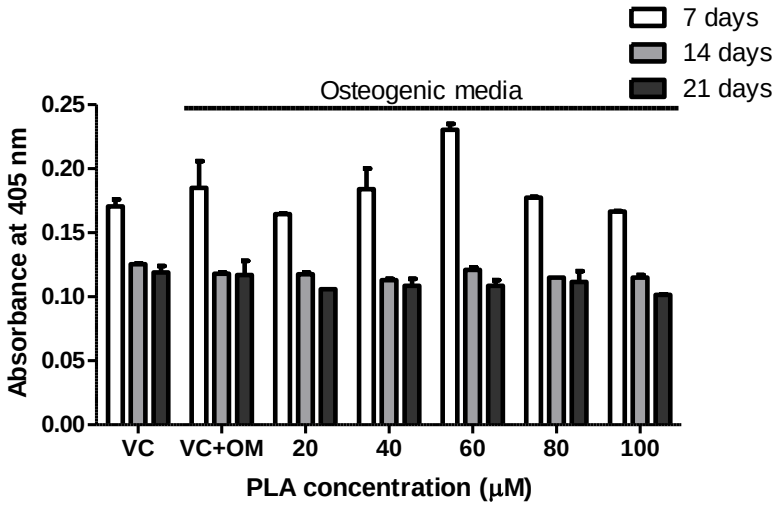

Figure 4.3. The effects of PLA on ALP activity in ASCs at 7, 14 and 21 days of differentiation. ASCs were seeded at 5 000 cells per cm^2 in a 48-well plate and allowed to attach overnight. Cells were exposed to a range of PLA concentrations (20, 40, 60, 80, 100 μM) in OM. Absorbance was measured at 405 nm. Data are representative of three independent biological repeats which were performed in duplicate.

4.2.2 The effects of palmitoleic acid on alkaline phosphatase activity in differentiated MG-63 osteosarcoma cells

MG-63 osteosarcoma cells were exposed to 20-100 μM PLA for up to 21 days in the presence of OM. Results revealed no significant changes in ALP activity at 7, 14, and 21 days in cells grown the presence of PLA when compared to the relevant VC+OM (Figure 4.4). ALP activity increased at 21 days compared to 7 and 14 days. This however was not statistically significant.

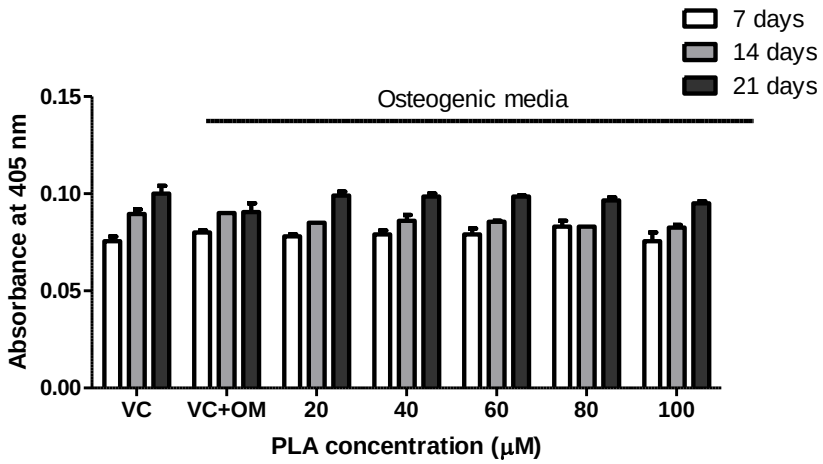

Figure 4.4. The effects of PLA on ALP activity in MG-63 cells at 7, 14 and 21 days of differentiation.

MG-63 were seeded at 10 000 cells per cm^2 in a 48-well plate and allowed to attach overnight. Cells were exposed to a range of PLA concentrations (20, 40, 60, 80, 100 μM) in OM. Absorbance was measured at 405 nm. Data are representative of three independent biological repeats which were performed in duplicate.

4.3 Mineralisation: Alizarin Red S staining

During bone formation, bone becomes mineralised in the late stages of differentiation. The effects of PLA on calcium mineralisation was assessed in ASCs and MG-63 osteosarcoma cells using an Alizarin Red S staining. Cells were differentiated and treated with various concentrations of PLA for 14 and 21 days. Thereafter, the staining was eluted and represented graphically.

4.3.1 The effects of palmitoleic acid on Alizarin Red S staining in differentiated adipose-derived stromal cells

ASCs were exposed to 20-100 μM PLA for 14 and 21 days in the presence of OM. No stained calcium nodules were observed at 14 days of differentiation (Figure 4.5). Darker red stained areas were seen in the VC, VC+OM, 80 μM and 100 μM PLA at 14 days. Morphological changes were seen at 14 days where cells in the VC were thinner and more spindle shaped. Cells exposed to OM and PLA were larger and less spindle shaped.

At 21 days of differentiation (Figure 4.6), again Alizarin Red S stained calcium nodules were not present. Darker red stained areas were present at 40-100 μM PLA.

Morphological changes were seen at 21 days of differentiation. In the VC, cells were smaller and more spindle shaped whereas in VC+OM and PLA treated cells, the cells were more round and larger in size.

The staining was eluted, measured by spectrophotometry and represented graphically (Figure 4.7). Results at 14 days showed the absorbance was not significantly increased between the VC+OM and PLA treated cells, however, there was an increase in the absorbance of the eluted dye at 20 μM, 60 μM and 80 μM PLA. This correlates with results seen in Figure 4.5 at 80 μM PLA.

At 21 days, the absorbance was increased at 20- 100 μM PLA, which was statistically significant ($p<0.05$) at 80 μM PLA compared to the VC+OM. This corresponds with darker stained areas which were observed in Figure 4.6. A trend was observed where there was an increase in the absorbance at 21 days compared to 14 days for 80 μM and 100 μM PLA.

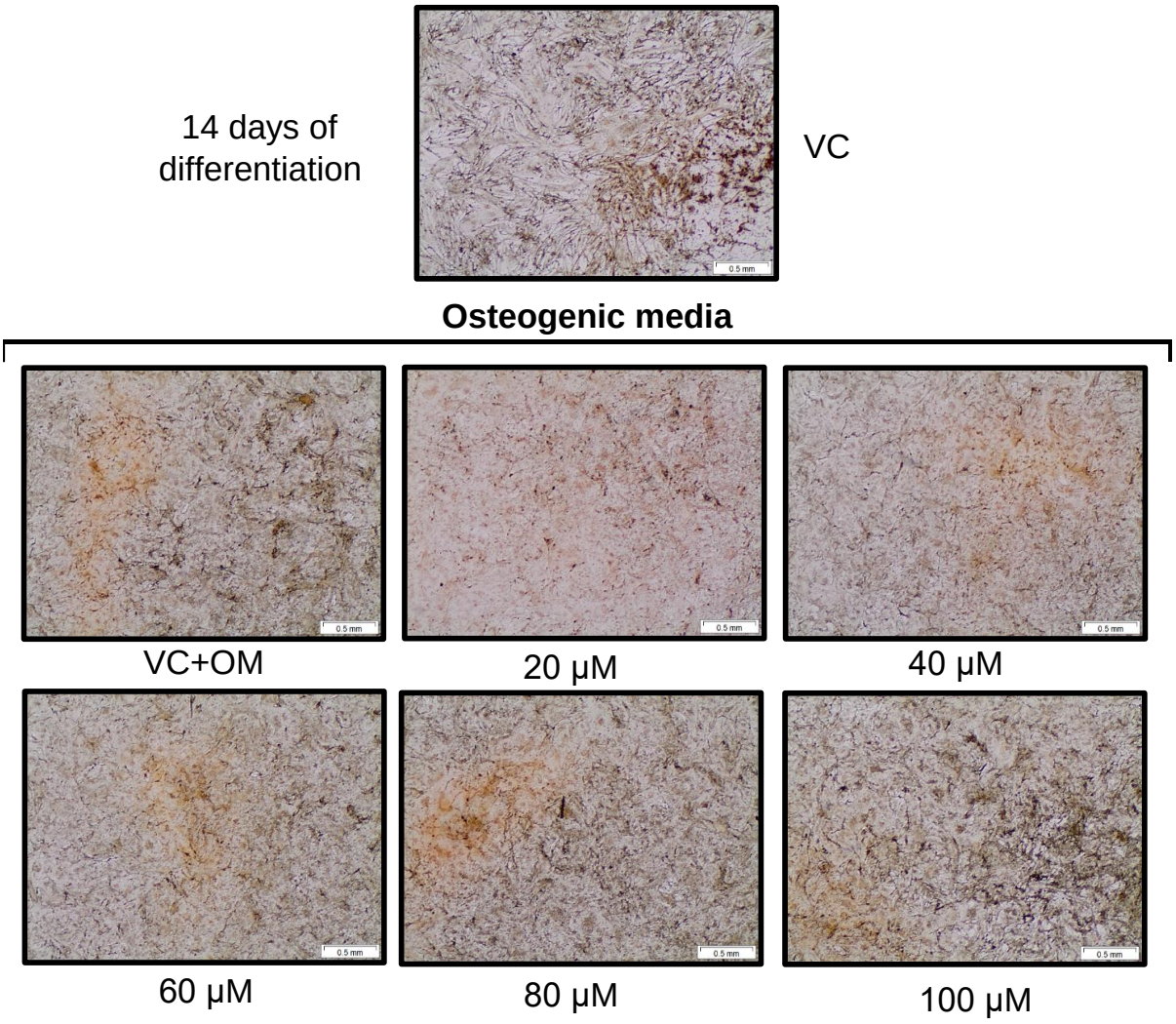

Figure 4.5. The effects of PLA on mineralisation in ASCs at 14 days of differentiation.

ASCs were seeded at 5 000 cells per cm^2 in a 48-well plate and allowed to attach overnight. Cells were exposed to a range of PLA concentrations (20, 40, 60, 80, 100 µM) in OM. Alizarin Red S staining was performed. Data are representative of three independent biological repeats which were performed in duplicate. Scale bar = 0.5 mm

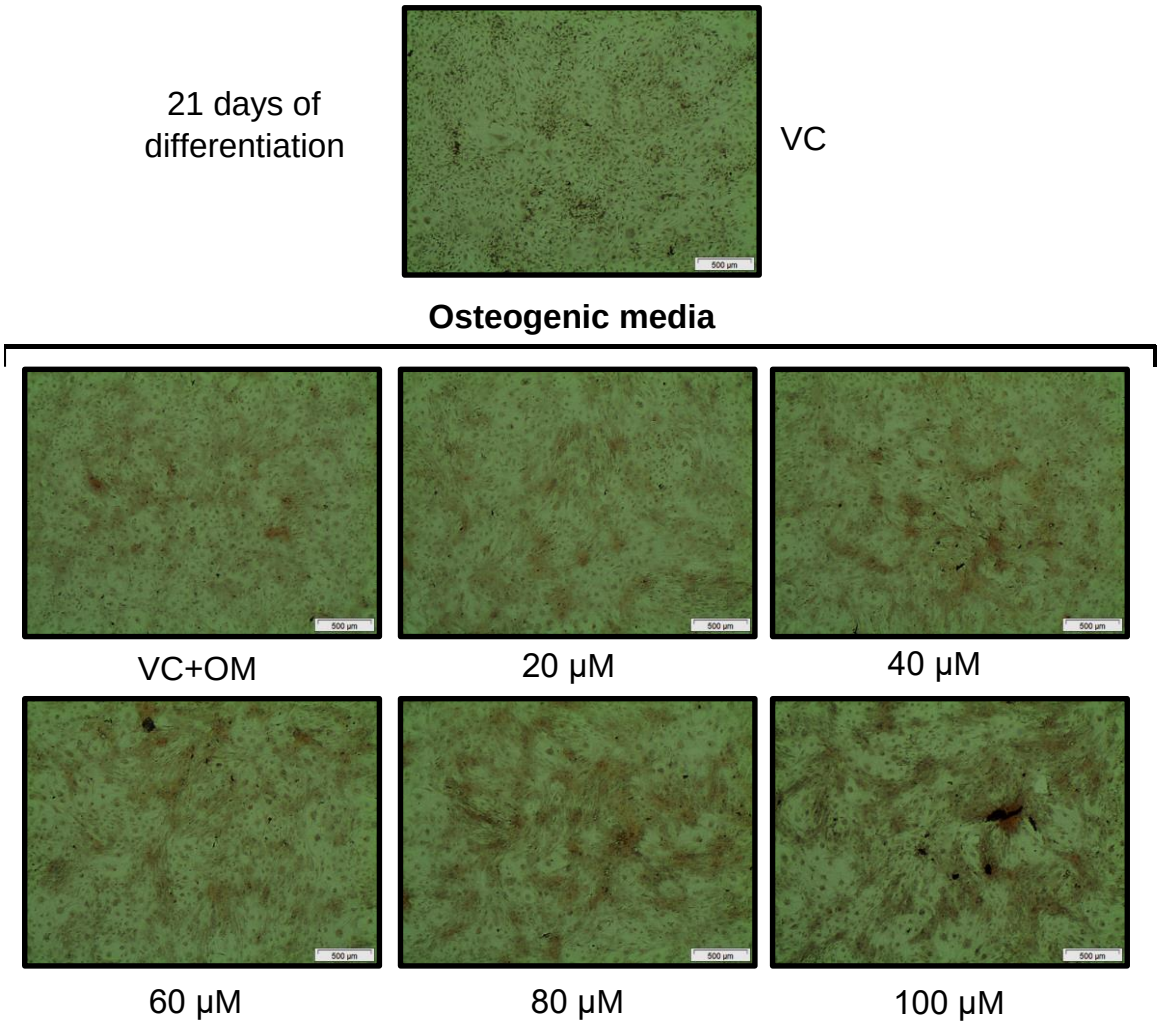

Figure 4.6. The effects of PLA on mineralisation in ASCs at 21 days of differentiation.

ASCs were seeded at 5 000 cells per cm^2 in a 48-well plate and allowed to attach overnight. Cells were exposed to a range of PLA concentrations (20, 40, 60, 80, 100 µM) in OM. Alizarin Red S staining was performed. Data are representative of three independent biological repeats which were performed in duplicate. Scale bar = 500 µm.

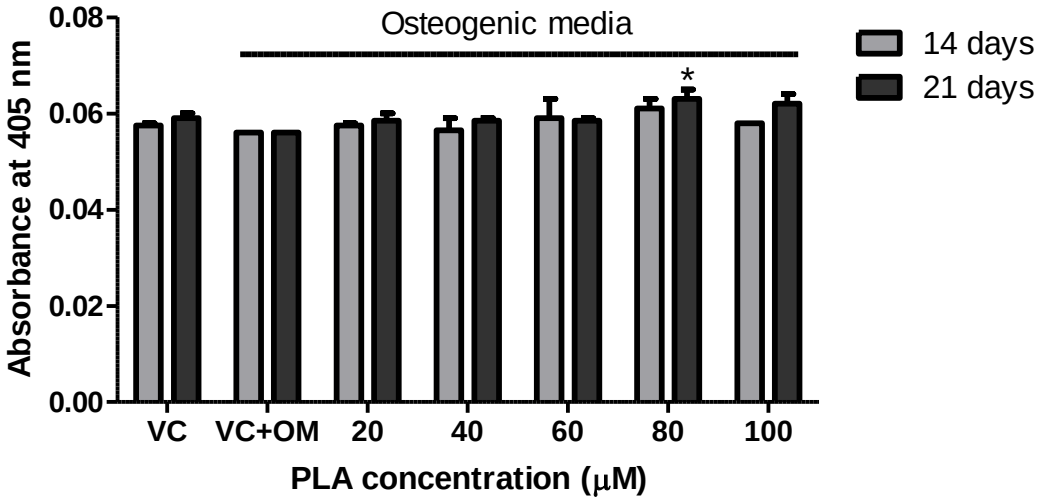

Figure 4.7. Quantification of Alizarin Red S staining elution in ASCs at 14 and 21 days of differentiation in the presence of PLA.

ASCs were seeded at 5 000 cells per cm^2 in a 48-well plate and allowed to attach overnight. Cells were exposed to a range of PLA concentrations (20, 40, 60, 80, 100 μM) in OM. Alizarin Red S staining was performed and the dye was eluted. Data are representative of three independent biological repeats which were performed in duplicate. *$p<0.05$ vs VC+OM.

4.3.2 The effects of palmitoleic acid on Alizarin Red S staining in differentiated MG-63 osteosarcoma cells

MG-63 osteosarcoma cells were exposed to 20-100 μM PLA for 14 and 21 days in the presence of OM. At 14 days, no calcium nodules were noticed (Figure 4.8). Darker stained areas were observed in the VC, 80 μM and 100 μM PLA compared to the VC+OM at 14 days. There were however morphological differences between the VC and cells treated with OM. In the VC, cells were irregular in shape and the border between each cell was still distinguishable. The cells treated with OM appeared more round and larger in size and cells were indistinguishable from one another as the borders between each cell was not visible.

At 21 days of differentiation, again no calcium nodules were observed (Figure 4.9). Darker stained areas were observed in the VC+OM, 20 μM, 40 μM and 100 μM PLA. There were morphological differences seen between the VC in the absence of OM and cells treated with OM. In the VC, cells were irregular in shape and the border between each cell was still distinguishable. There were also more spaces visible between the cells. The cells treated with OM appeared larger and more round and

cells were indistinguishable from one another as the borders between each cell was not visible, thus cells were closer to one another.

The dye was eluted, measured spectrophotometrically and represented graphically in Figure 4.10. At 14 days, no significant changes were seen after PLA exposure compared to the VC+OM. At 21 days, the absorbance was increased at 20-80 μM PLA but not significantly, compared to VC+OM. A trend was observed where there was an increase in the absorbance at 21 days compared to 14 days for VC+OM, 20 μM, 40 μM, 60 μM, 80 μM and 100 μM PLA.

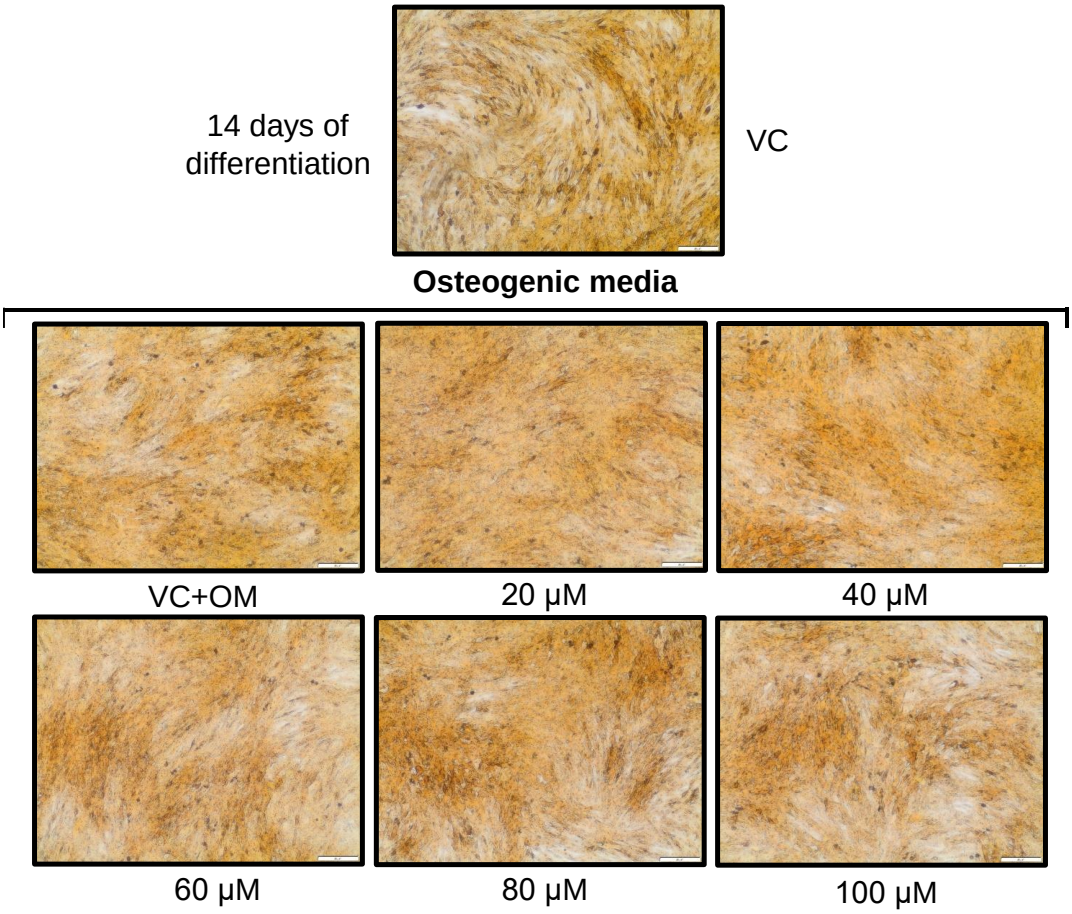

Figure 4.8. The effects of PLA on mineralisation in MG-63 cells at 14 days of differentiation. MG-63 cells were seeded at 10 000 cells per cm^2 in a 48-well plate and allowed to attach overnight. Cells were exposed to a range of PLA concentrations (20, 40, 60, 80, 100 µM) in OM. Alizarin Red S staining was performed. Data are representative of three independent biological repeats which were performed in duplicate. Scale bar = 200 µm.

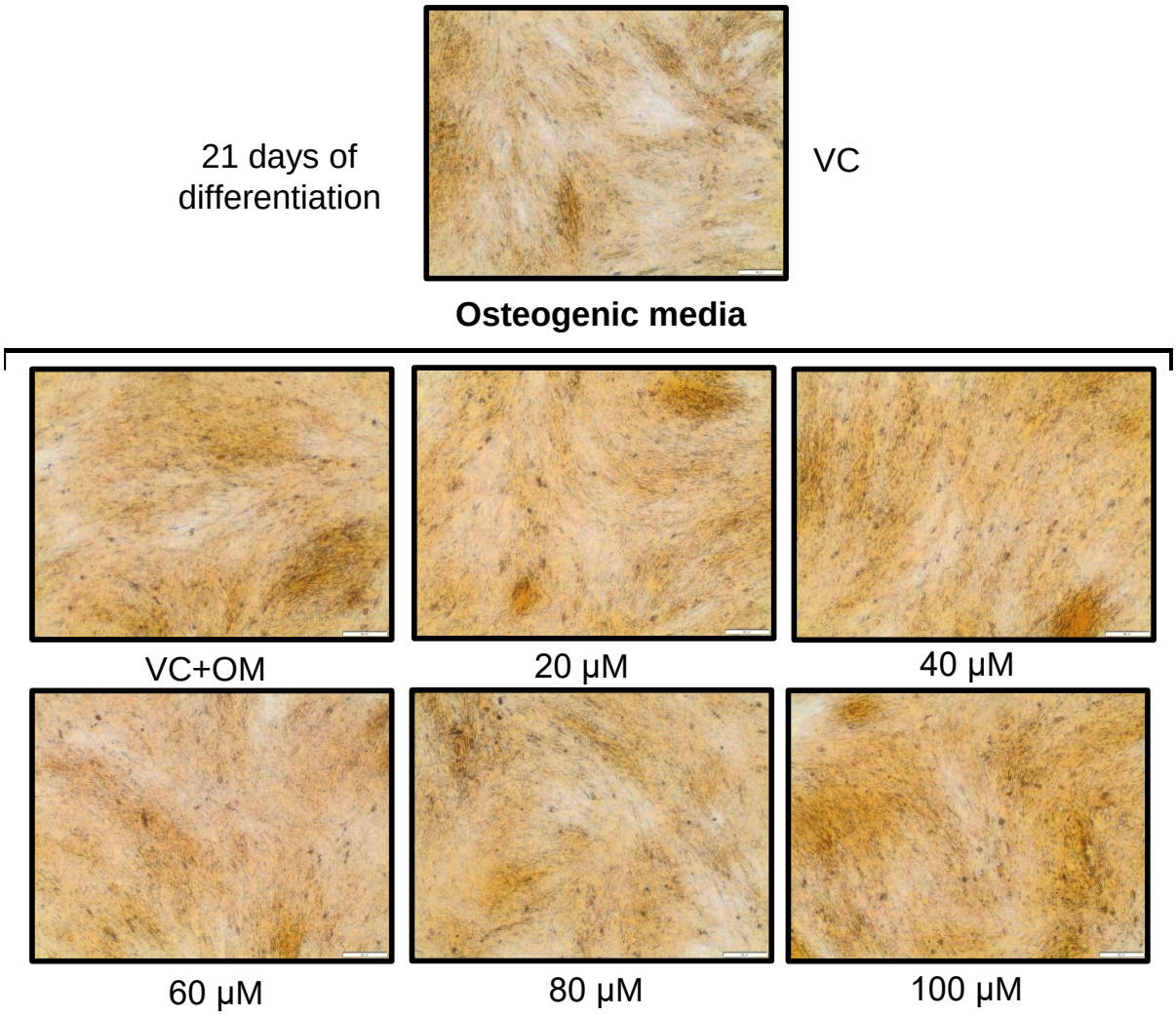

Figure 4.9. The effects of PLA on mineralisation in MG-63 cells at 21 days of differentiation. MG-63 cells were seeded at 10 000 cells per cm^2 in a 48-well plate and allowed to attach overnight. Cells were exposed to a range of PLA concentrations (20, 40, 60, 80, 100 µM) in OM. Alizarin Red S staining was performed. Data are representative of three independent biological repeats which were performed in duplicate. Scale bar = 200 µm.

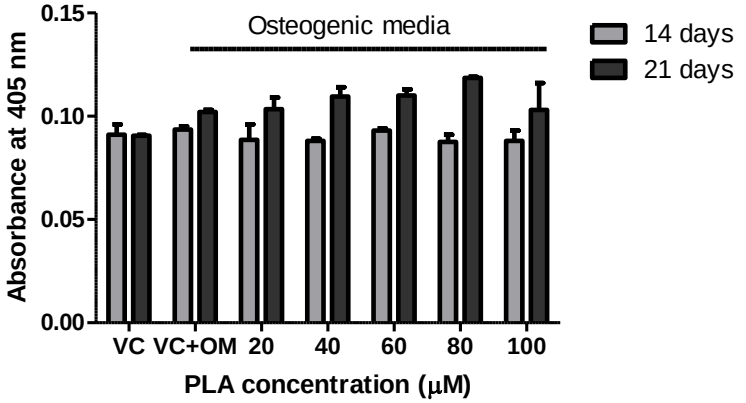

Figure 4.10. The effects of PLA on Alizarin Red S staining elution in MG-63 cells at 14 and 21 days of differentiation.

MG-63 cells were seeded at 10 000 cells per cm^2 in a 48-well plate and allowed to attach overnight. Cells were exposed to a range of PLA concentrations (20, 40, 60, 80 and 100 μM) in OM. Alizarin Red S staining was performed and the dye was eluted. Data are representative of three independent biological repeats which were performed in duplicate.

4.4 Gene expression: quantitative polymerase chain reaction

The effects of PLA on the gene expression of early osteogenic markers was determined in ASCs and MG-63 osteosarcoma cells using qPCR. Analysis of the mRNA expression for the following genes in differentiating osteoblasts were performed: ALP, RUNX2, Osx, OCN, OPG and RANKL. Cells were differentiated and treated with 100 μM PLA for 7 and 14 days to assess early osteoblast-specific gene expression. Late osteogenic markers such as ALP, OCN and BSP, and adipogenesis marker PPAR-γ, were not included as it was difficult to extract mRNA from the secreted matrix at 21 days of differentiation. For this study, the effect of PLA on gene expression was determined at 100 μM PLA only due to time and cost limitations.

4.4.1 The effects of palmitoleic acid on gene expression in early differentiating adipose-derived stromal cells

Comparisons were drawn between the cells grown in the absence of OM (VC) and the VC in the presence of OM (VC+OM) at 7 days of differentiation. ALP was significantly decreased ($p<0.05$) in the VC+OM compared to the VC (Figure 4.11A). RUNX2 expression showed an increase in the VC+OM compared to the VC (Figure 4.11B). Osx, RANKL and the OPG/RANKL ratio was decreased in the VC+OM compared to

the VC but none of these were statistically significant (Figure 4.11C, F and G). OPG showed a significant decrease ($p<0.01$) in the VC+OM compared to the VC (Figure 4.11E).

ASCs were exposed to 100 µM PLA for 7 and 14 days in the presence of OM. At 7 days of differentiation, ALP was significantly increased ($p<0.01$) after 100 µM PLA compared to the cells treated with VC+OM (Figure 4.11A). RUNX2 expression was increased after 100 µM PLA treatment compared to the VC+OM (Figure 4.11B). This change was not statistically significant. Osx and OCN expression showed a minor increase after 100 µM PLA exposure compared to the VC+OM but it was also not significant (Figure 4.11C and D). OPG showed a small decrease in the gene expression after 100 µM PLA exposure compared to the VC+OM, however, no statistical significance was observed (Figure 4.11E). RANKL gene expression was significantly decreased ($p<0.05$) after 100 µM PLA exposure compared to the VC+OM (Figure 4.11F) while the OPG/RANKL ratio showed an increase after 100 µM PLA treatment compared to the VC+OM. The trends were however not statistically significant.

At 14 days, comparisons drawn between the VC+OM and the VC revealed increases in the gene expression of ALP, RUNX2, Osx and OPG/RANKL ratio but these changes were not significant (Figure 4.12A, B, C and G). Gene expression of OCN, OPG and RANKL, on the other hand, decreased in the VC+OM compared to the VC (Figure 4.12D, E and F), although these trends were also not statistically significant.

At 14 days of differentiation, ALP gene expression was significantly increased ($p<0.05$) after 100 µM PLA exposure compared to the VC+OM (Figure 4.12A). RUNX2, Osx, OCN, OPG, RANKL and the OPG/RANKL ratio showed increases in gene expression after 100 µM PLA exposure compared to the VC+OM (Figure 4.12B, C, D, E, F and G), again none of these changes were statistically significant.

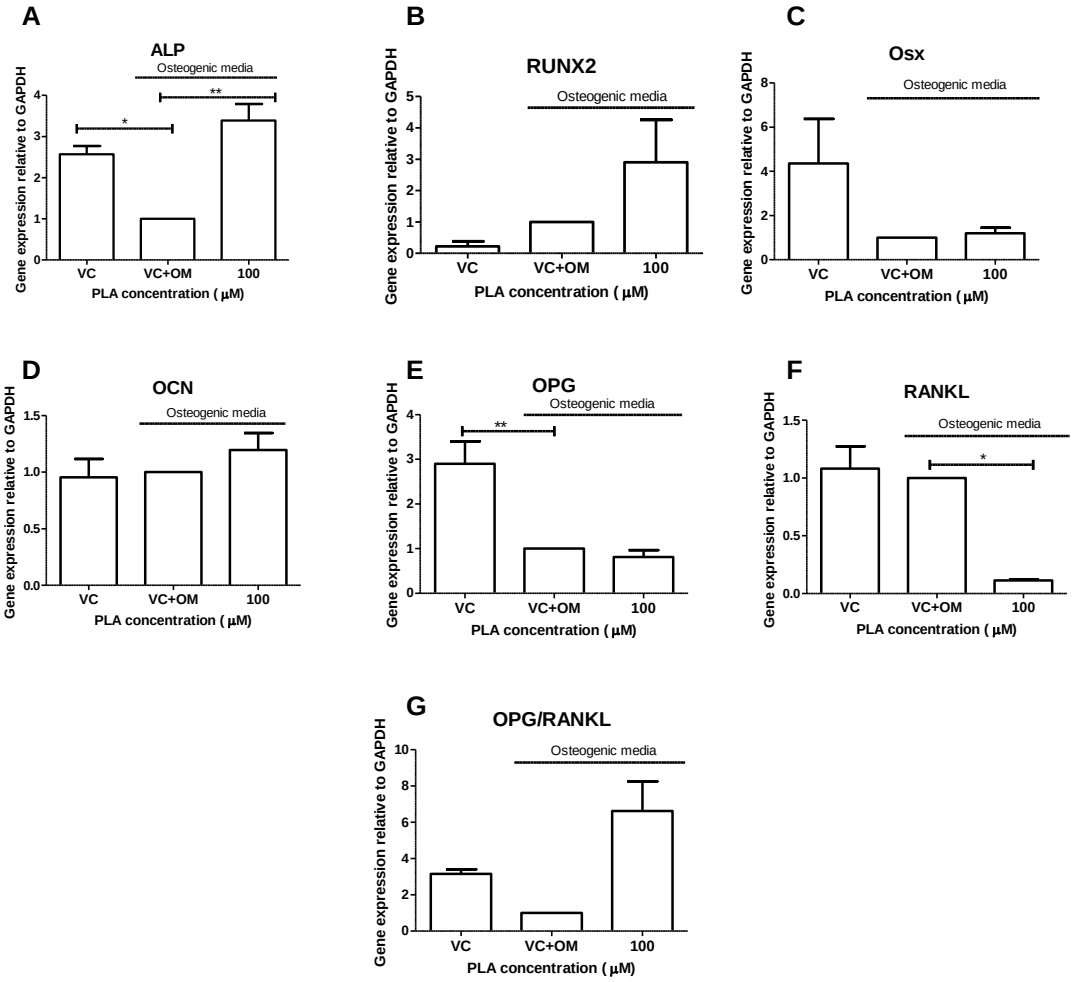

Figure 4.11. The effects of PLA on gene expression in ASCs at 7 days of differentiation.

ASCs were seeded at 5 000 cells per cm^2 in a 48-well plate and allowed to attach overnight. Cells were exposed to a PLA concentration of 100 µM in OM for 14 days. Gene expression for ALP, RUNX2, Osx, OCN, OPG and RANKL was determined using qPCR. Data was analysed using the $2^{-\Delta\Delta CT}$ method with GAPDH used as the housekeeping gene. Data are representative of three independent biological repeats which were performed in duplicate. *p<0.05 vs VC+OM and **p<0.01 vs VC+OM.

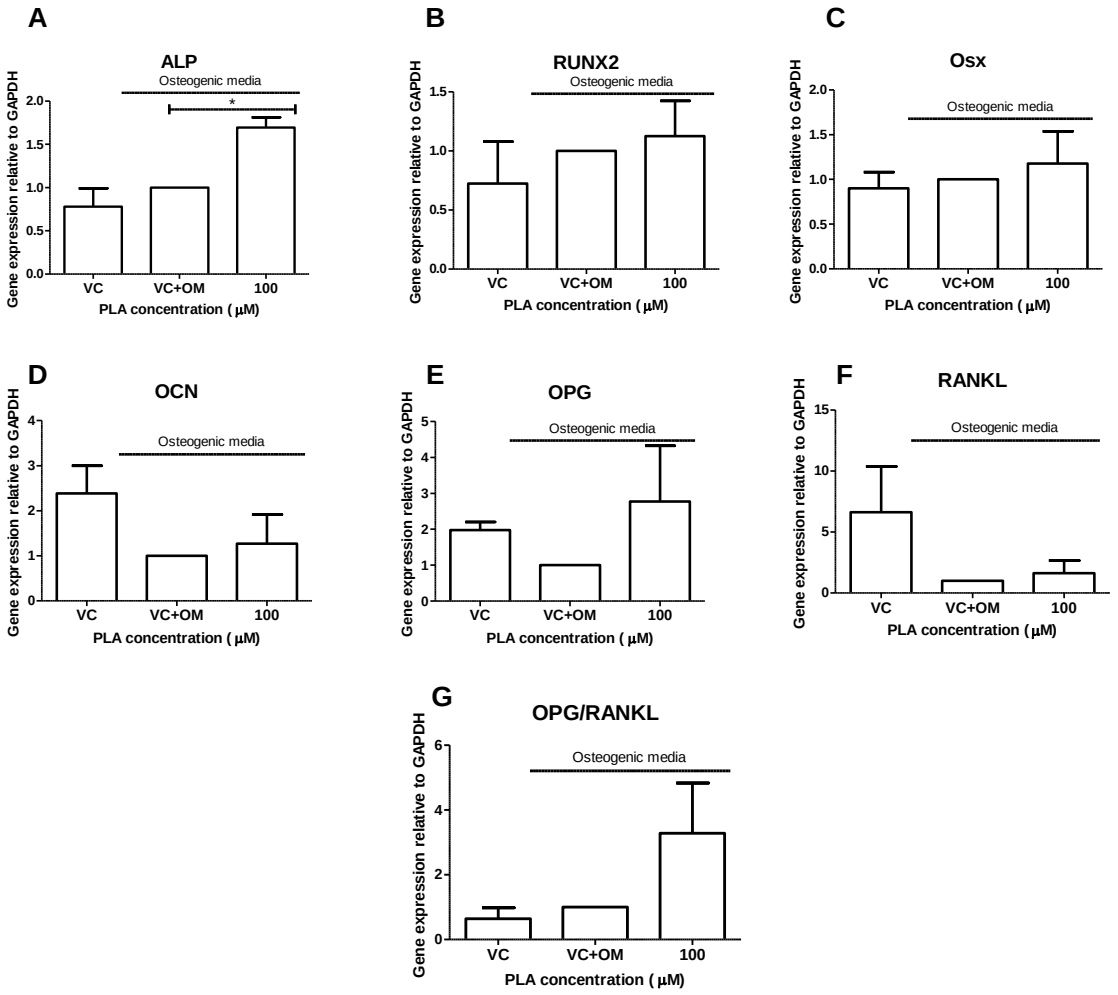

Figure 4.12. The effects of PLA on gene expression in ASCs at 14 days of differentiation.
ASCs were seeded at 5 000 cells per cm^2 in a 48-well plate and allowed to attach overnight. Cells were exposed to a PLA concentration of 100 μM in OM for 14 days. Gene expression for ALP, RUNX2, Osx, OCN OPG, and RANKL was determined using qPCR. Data was analysed using the $2^{-\Delta\Delta CT}$ method with GAPDH used as the housekeeping gene. Data are representative of three independent biological repeats which were performed in duplicate. *p<0.05 vs VC+OM.

4.4.2 The effects of palmitoleic acid on gene expression in early differentiating MG-63 osteosarcoma cells

Gene expression of ALP ($p<0.05$), RUNX2 ($p<0.001$), OCN ($p<0.001$) and RANKL ($p<0.01$) were significantly increased in the VC+OM compared to the VC at 7 days (Figure 4.13A, B, D and F). Trends in Osx and OPG gene expression revealed decreases in the VC+OM compared to the VC (Figure 4.13C and E), however, the

trends were not statistically significant. The OPG/RANKL ratio showed a significant decrease ($p<0.001$) in the VC+OM compared to the VC (Figure 4.13G).

MG-63 osteosarcoma cells were exposed to 100 µM PLA for 7 and 14 days of differentiation in the presence of OM. At 7 days of differentiation, ALP, Osx and the OPG/RANKL ratio showed increases in gene expression after 100 µM PLA exposure compared to the VC+OM (Figure 4.13A, C and G). These changes were however not statistically significant. RUNX2 and OCN gene expression was significantly reduced ($p<0.05$) after 100 µM PLA exposure compared to the VC+OM (Figure 4.13B and D). OPG and RANKL gene expression showed a decrease after 100 µM PLA exposure compared to the VC+OM (Figure 4.13E and F). These trends were not statistically significant.

A significant increase ($p<0.001$) in ALP, RUNX2, Osx and RANKL gene expression was also observed after 14 days of differentiation when comparing the VC+OM to the VC (Figure 4.14A, B, C and F). OCN gene expression showed an increase when comparing the VC+OM to VC (Figure 4.14D) while OPG gene expression showed an increase in the VC+OM when compared to the VC (Figure 4.14E), however, these were not statistically significant. The OPG/RANKL ratio was significantly decreased ($p<0.001$) in the VC+OM compared to the VC (Figure 4.14G).

At 14 days of differentiation, RUNX2 ($p<0.05$), ALP, Osx, OCN and RANKL ($p<0.001$) were significantly reduced after 100 µM PLA exposure compared to VC+OM (Figure 4.14A, B, C, D and F). OPG expression was decreased after 100 µM PLA exposure compared to the VC+OM but this was not significant (Figure 4.14E). The OPG/RANKL ratio indicated an increase in gene expression after 100 µM PLA exposure compared to the VC+OM (Figure 4.14G) but this was not statistically significant.

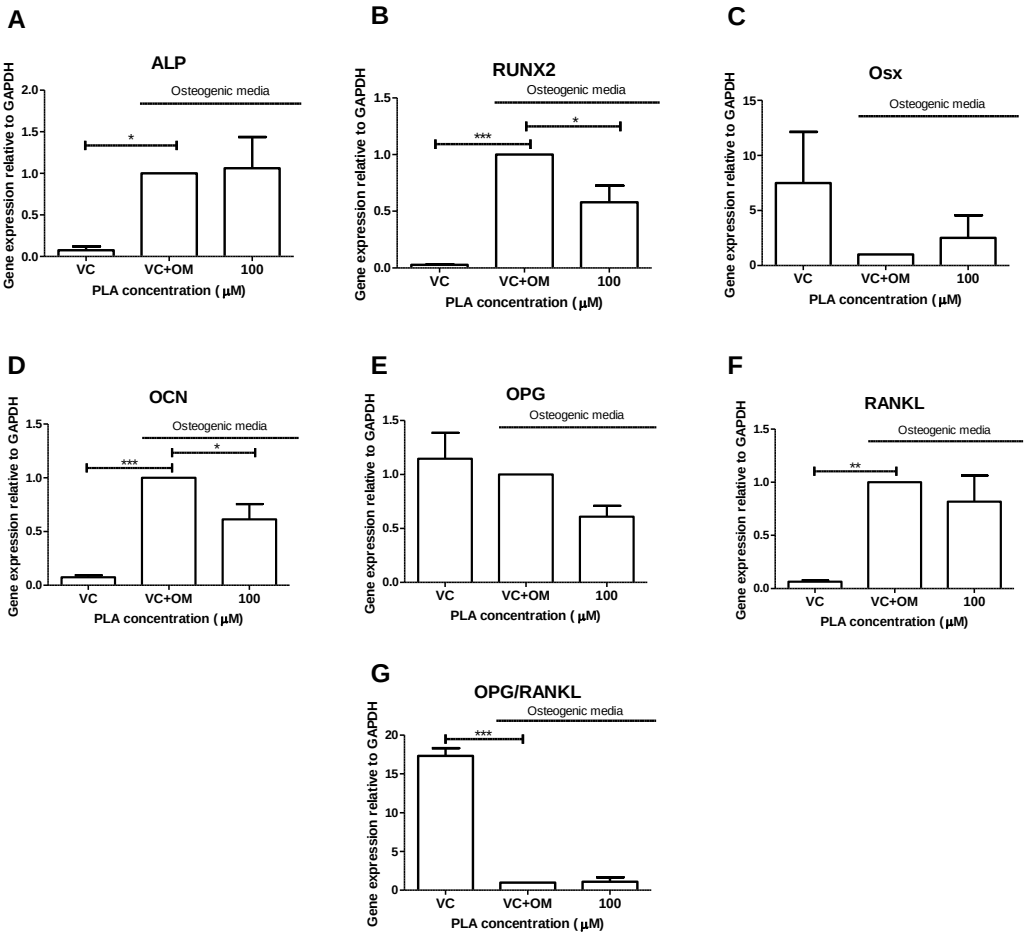

Figure 4.13. The effects of PLA on gene expression in MG-63 cells at 7 days of differentiation. MG-63 osteosarcoma cells were seeded at 10 000 cells per cm^2 in a 48-well plate and allowed to attach overnight. Cells were exposed to a PLA concentration of 100 μM in OM for 7 days. Gene expression for ALP, RUNX2, Osx, OCN, OPG and RANKL was determined using qPCR. Data was analysed using the $2^{-\Delta\Delta CT}$ method after being made relative to GAPDH. Data are representative of three independent biological repeats which were performed in duplicate. *p<0.05 vs VC+OM, **p<0.01 vs VC+OM, ***p<0.001 vs VC+OM.

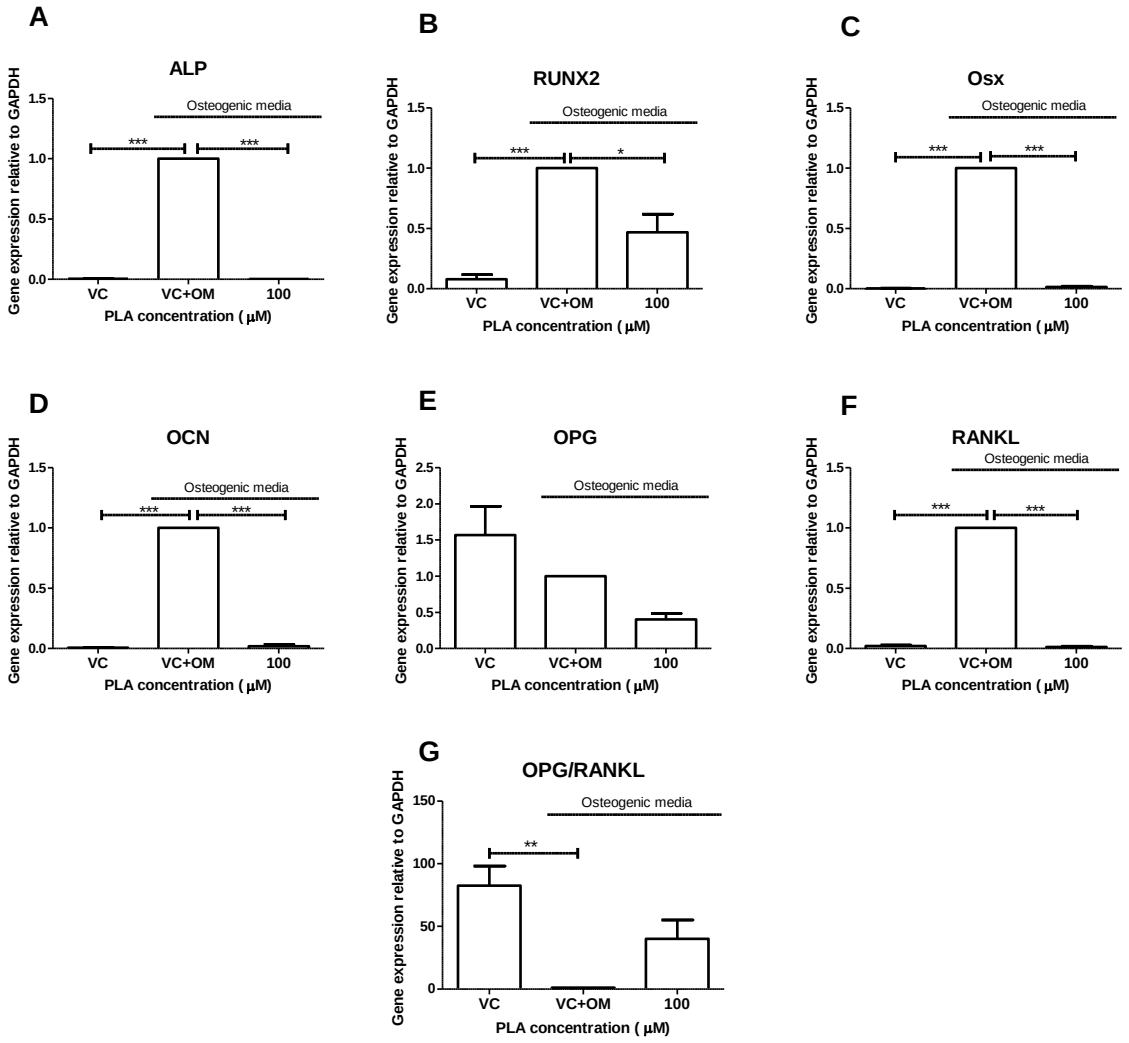

Figure 4.14. The effects of PLA on gene expression in MG-63 cells at 14 days of differentiation. MG-63 osteosarcoma cells were seeded at 10 000 cells per cm^2 in a 48-well plate and allowed to attach overnight. Cells were exposed to a PLA concentration of 100 µM in OM for 14 days. Gene expression for ALP, RUNX2, Osx, OCN, OPG and RANKL, was determined using qPCR. Data was analysed using the $2^{-\Delta\Delta CT}$ method after being made relative to GAPDH. Data are representative of three independent biological repeats which were performed in duplicate. *p<0.05 vs VC+OM, **p<0.01 vs VC+OM, ***p<0.001 vs VC+OM.

4.5 Protein expression: Western blotting

The effects of PLA on the protein expression was determined in MG-63 osteosarcoma cells using western blotting. The expression of the following MAPK markers were determined in differentiating osteoblasts: ERK and pERK. Western blotting on the ASCs were not performed as these cells are large and slow growing thus due to time constraints, not enough cells could be grown to perform a western blot on them.

4.5.1 The effects of palmitoleic acid on ERK activation in early differentiating MG-63 osteosarcoma cells

MG-63 osteosarcoma cells were exposed to 100 μM PLA for 3 hours, 6 hours and 24 hours in the presence of OM. Results revealed thicker bands in pERK expression at 6 hours compared to 3 and 24 hours (Figure 4.15). ERK band intensities were lower for ERK at 3 hours compared to 6 and 24 hours. The band intensities were quantified with ImageJ software and relative pERK/ERK expression was determined.

The pERK/ERK expression at 3 hours of differentiation showed a minor increase after exposure to 100 μM PLA compared to the VC+OM (Figure 4.16A) while the VC+OM pERK/ERK expression was decreased compared to VC, however, these trends were not statistically significant. At 6 hours, there was an increase after exposure to 100 μM PLA compared to the VC+OM while the VC+OM was decreased compared to the VC although these changes were again not statistically significant (Figure 4.16B). At 24 hours, pERK/ERK expression was decreased after exposure to 100 μM PLA compared to the VC+OM while the VC+OM was reduced compared to the VC (Figure 4.16C). These trends were not statistically significant.

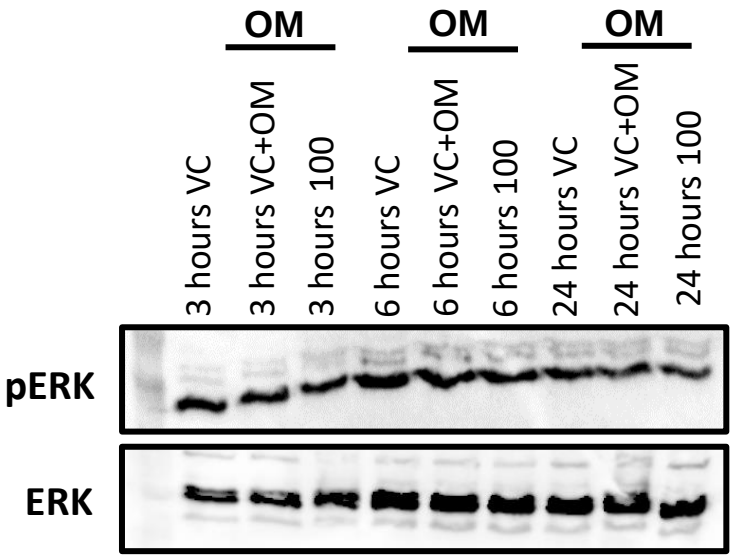

Figure 4.15. The effects of PLA on relative pERK/ERK protein expression in MG-63 cells at 3, 6 and 24 hours.

MG-63 were seeded at 10 000 cells per cm^2 in a 6-well plate and allowed to attach overnight. Cells were exposed to 100 µM PLA and OM. Western blotting was performed. The thickness of the bands resemble the relative protein expression. Data are representative of three independent biological repeats.

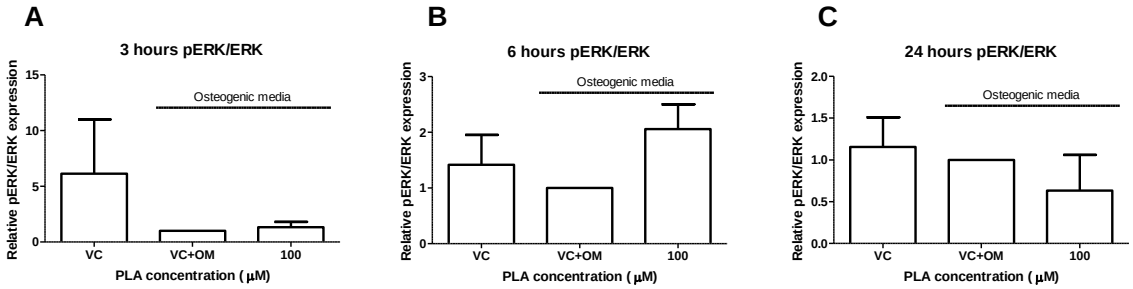

Figure 4.16. The effects of PLA on relative pERK/ERK protein expression in MG-63 cells at 3, 6 and 24 hours.

MG-63 were seeded at 10 000 cells per cm^2 in a 6-well plate and allowed to attach overnight. Cells were exposed to 100 µM PLA and OM. Western blotting was performed and blots were analysed using ImageJ software to determine relative densities. Data are representative of three independent biological repeats.

4.6 Oil Red O staining

The effects of PLA on lipid accumulation was assessed on ASCs and MG-63 osteosarcoma cells using an Oil Red O staining. Cells were differentiated into osteoblasts and treated with various concentrations of PLA for up to 21 days. Thereafter, Oil Red O staining was used to stain for lipids to ensure the cells were not differentiating into adipocytes. This staining was then eluted and represented graphically.

4.6.1 The effects of palmitoleic acid on Oil Red O staining in differentiated adipose-derived stromal cells

ASCs were exposed to 20, 40, 60, 80 and 100 μM PLA for up to 21 days in the presence of OM. Results revealed no significant increases in the presence of lipid droplets at 21 days when compared to the VC+OM (Figure 4.17). Morphological differences were seen between the VC and cells exposed to OM. In the VC, cells were triangular shaped with long thin fibres branching out to neighbouring cells. In the VC+OM, cells were more round in shape with almost no fibres protruding from the cells. As the concentration of PLA increased, cells became larger with more fibres branching out to neighbouring cells. The cells created a monolayer at 100 μM PLA. In the positive control, lipid accumulation can be seen in the red-stained intracellular lipid droplets which indicates that the cells could be adipocytes.

The dye was eluted using 100% isopropyl alcohol and represented graphically. Results showed no significant increases in the absorbance of the dye although there was an increase in the absorbance in cells treated with 20-100 μM PLA (Figure 4.18) but this was not statistically significant.

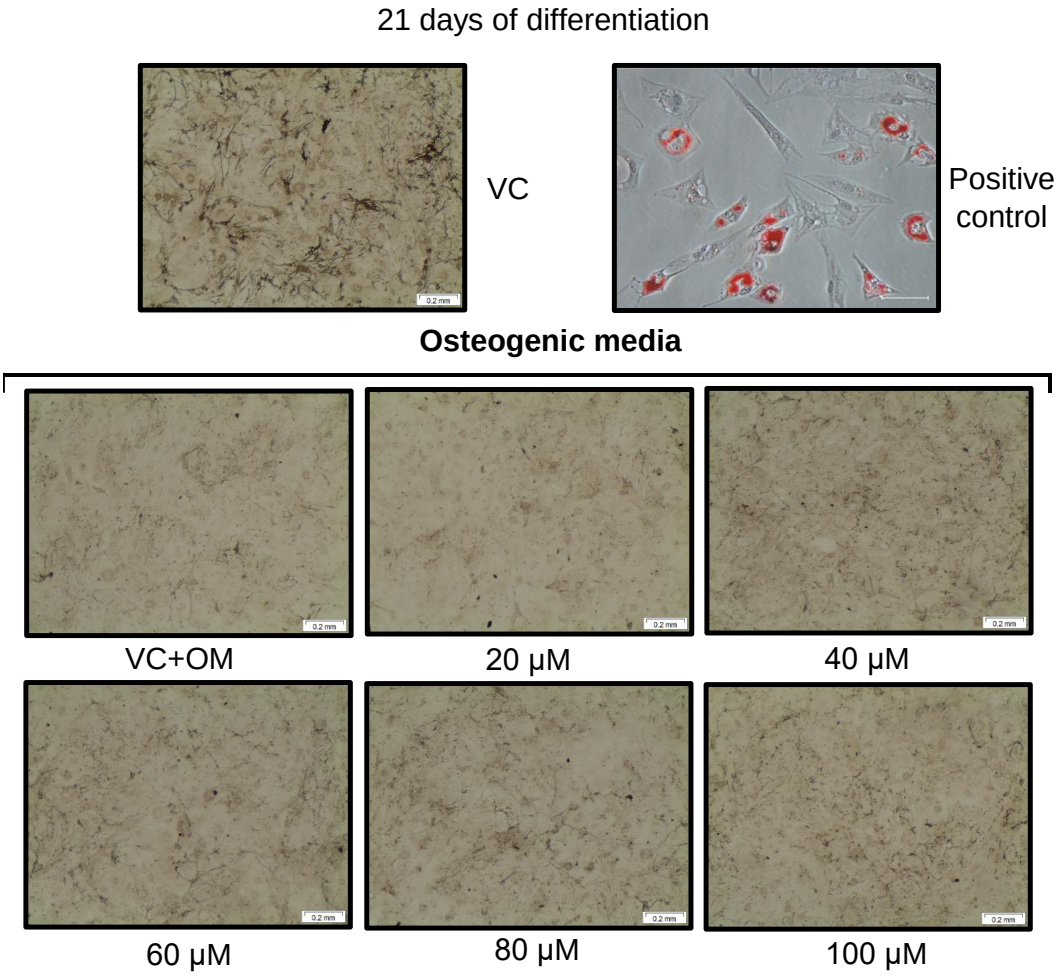

Figure 4.17. The effects of PLA on adipogenesis in ASCs at 21 days of differentiation.
ASCs were seeded at 5 000 cells per cm^2 in a 48-well plate and allowed to attach overnight. Cells were exposed to a range of PLA concentrations (20, 40, 60, 80, 100 µM) in OM. Oil Red O staining was performed. Data are representative of three independent biological repeats which were performed in duplicate. Scale bar = 0.2 mm. Copyright for positive control image obtained from Copyright Clearance Centre RightsLink (See appendix C). Image taken from Martella *et al*.[233]

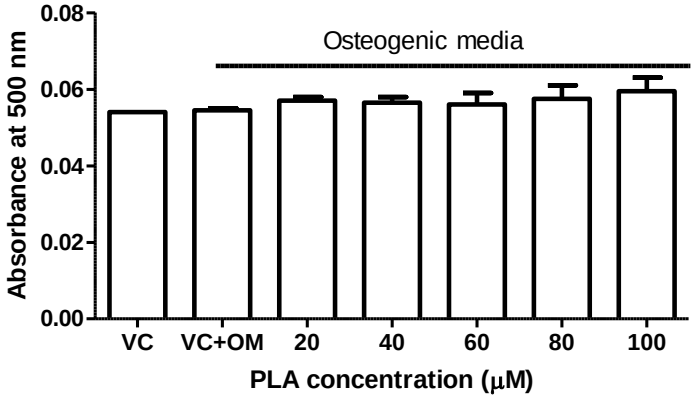

Figure 4.18. The effects of PLA on Oil Red O staining elution in ASCs at 21 days of differentiation. ASCs were seeded at 5 000 cells per cm^2 in a 48-well plate and allowed to attach overnight. Cells were exposed to a range of PLA concentrations (20, 40, 60, 80, 100 μM) in OM. Oil Red O staining was performed and the dye was eluted. Data are representative of three independent biological repeats which were performed in duplicate.

4.6.2 The effects of palmitoleic acid on Oil Red O staining in differentiated MG-63 osteosarcoma cells

MG-63 osteosarcoma cells exposed to 20-100 μM PLA for 21 days in the presence of OM showed no intracellular lipid droplets (Figure 4.19). Morphological changes were seen in the VC compared to the VC+OM and cells exposed to PLA. In the VC, cells are irregular shaped with some dark stained areas. In the VC+OM and PLA treated cells, the cell border was more visible with some round cells present in between elongated irregular shaped cells. Darker stained areas can be seen at 60 μM and 100 μM PLA. At 80 μM and 100 μM PLA, the red dye can be seen which indicates there may be lipid droplets present at these high concentrations.

The dye was eluted using 100% isopropyl alcohol and represented graphically. The absorbance increased at 20-100 μM PLA. At 20-80 μM PLA, the increase was not statistically significant (Figure 4.20), however, at 100 μM PLA the increase was significant ($p<0.05$).

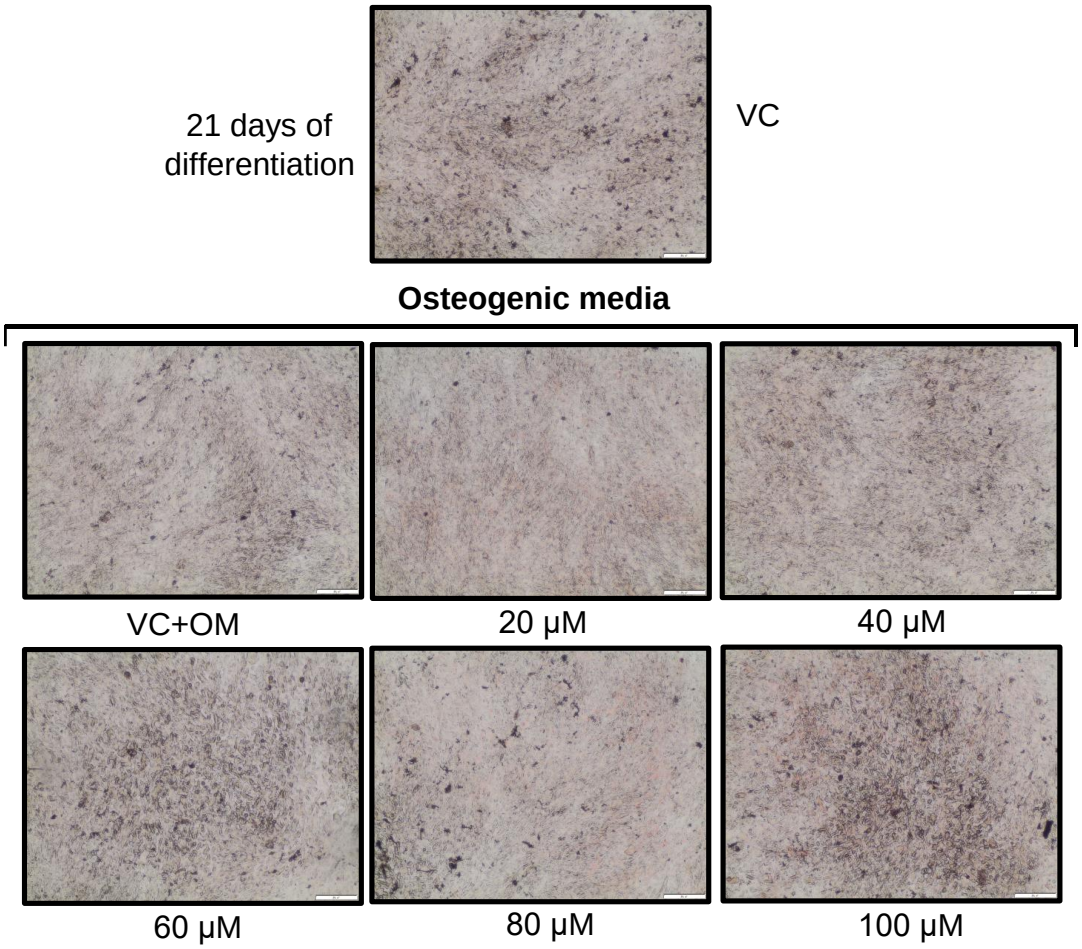

Figure 4.19. The effects of PLA on Oil Red O staining in MG-63 cells at 21 days of differentiation. MG-63 cells were seeded at 10 000 cells per cm^2 in a 48-well plate and allowed to attach overnight. Cells were exposed to a range of PLA concentrations (20, 40, 60, 80, 100 µM) in OM. Oil Red O staining was performed. Data are representative of three independent biological repeats which were performed in duplicate. Scale bar = 200 µm.

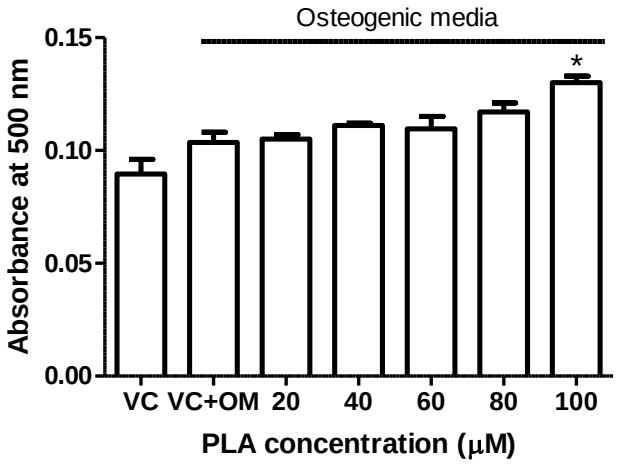

Figure 4.20. The effects of PLA on adipogenesis in MG-63 cells at 21 days of differentiation. MG-63 cells were seeded at 10 000 cells per cm^2 respectively in a 48-well plate and allowed to attach overnight. Cells were exposed to a range of PLA concentrations (20, 40, 60, 80, 100 μM) in OM. Oil Red O staining was performed and the dye was eluted. Data are representative of three independent biological repeats which were performed in duplicate. *$p<0.05$ vs VC+OM.

Chapter 5

Discussion

Osteoporosis and other bone fragility disorders can have a major impact on the quality of life in the elderly. UFA has been demonstrated to have a beneficial effect on bone.[9] PLA has previously been proven to reduce osteoclast activity thereby exerting a protective effect on bone.[12] The role of PLA on osteoblast differentiation has not yet been determined.

In this *in vitro* study, ASCs and MG-63 osteosarcoma cells were differentiated into osteoblast-like cells using OM and exposed to PLA, an omega-7 MUFA, at various concentrations. The effects were evaluated on osteoblast specific parameters such as ALP activity, calcium mineralisation, gene expression and activation of the ERK pathway. Thereafter, the cells were analysed for the presence of lipid deposits present in adipocytes. The purpose of this study was to determine the role of PLA in osteoblasts and its effect on the ERK pathway involved in early osteoblast differentiation. The results will be discussed and compared to previous *in vitro* studies.

5.1 Overview of research findings

Resazurin assay for cell viability

Cell viability assay is a crucial method used to assess whether a compound affects the mitochondrial function and thus metabolic activity of the cells under investigation. The cell viability of undifferentiated ASCs and MG-63 osteosarcoma cells were assessed after exposure to PLA at various concentrations, using a resazurin assay. Cells were treated for 24 hours and showed no significant changes in cell viability at 20-100 µM PLA exposure for undifferentiated ASCs (Figure 4.1) and MG-63 osteosarcoma cells (Figure 4.2).

In 2017, van Heerden *et al*., in our laboratory, reported that PLA had no cytotoxic effects on RAW264.7 murine macrophages after 24 hours of exposure at concentrations of 20-100 µM.[12] In a rat pancreatic β-cell line, PLA was also demonstrated to have non-toxic effects at 250 µM after 18-72 hours of exposure.[234]

Our current study supports results obtained in these studies as PLA had no effect on the metabolic activity of the ASCs and MG-63 osteosarcoma cells at the tested concentrations.

Alkaline phosphatase activity

ALP is a biochemical marker of osteoblasts. At an alkaline pH, this enzyme catalyses the hydrolysis of phosphate esters.[235] There are three isoenzymes of ALP in humans: tissue non-specific, placental and intestinal ALP. ALP released by osteoblasts is regarded as tissue non-specific ALP.[235] In this current study, an ALP activity assay was used to colorimetrically measure the amount of ALP enzyme released from the cells after exposure to PLA in osteoblasts at 14 and 21 days of differentiation. This is achieved by measuring the hydrolysis of *p*-nitrophenylphosphate into *p*-nitrophenol.[236] As this only measures the amount of ALP released by the cells, intracellular gene expression of ALP was also measured using qPCR.

In the ASCs, ALP activity levels measured the highest at 7 days and reached a peak at 60 µM PLA (Figure 4.3). At 14 and 21 days, ALP activity remained constant when compared to that of the VC+OM.

In a study by Weinzierl *et al*., the potential of human ASCs differentiating into osteoblasts was determined to be up until the 3rd passage. In their study, ASCs were differentiated for up to 6 weeks and ALP activity was assessed. Results showed that ALP was increased after the 3rd and 5th week of differentiation, however, the increase was less distinct. The age of the donors had no effect on the osteogenic potential of the ASCs.[237] The lack of ALP activity in the present study could possibly be attributed to the differentiation time and the passage of the cells, as in our study, ASCs were differentiated for up to 3 weeks and were used up until a passage of 5.

James *et al*., describes a method to stain ALP in murine mesenchymal cells which was normalised to total protein content. The stain used is a diazonium salt solution comprised of fast violet blue salt and a 4% napthol AS-MX phosphate alkaline solution, where alkaline phosphatase positive cells were stained purple.[236] It may therefore be more useful to compare ALP activity to total protein expressed by the cells.

In the MG-63 osteosarcoma cells, no changes in ALP activity was observed at 7 and 14 days when compared to the corresponding VC+OM (Figure 4.4). ALP activity increased at 21 days compared to 7 and 14 days, which was however not statistically significant. A study by Czekanska *et al*., reported that ALP levels reached a peak after 28 days of differentiation in MG-63 osteosarcoma cells[238], which may explain why no significant increases were seen at 7-21 days of differentiation in our study.

Calcium mineralisation

There are multiple methods which can be used to determine mineralisation. This can be achieved for instance through fluorescence staining[239], incorporation of Alizarin Red S staining[240] or Von Kossa staining[241]. Alizarin Red S is considered a suitable method as the staining can be eluted and assayed thereafter.[226] Gregory *et al*., found 10% acetic acid was more sensitive than cetylpyridinium chloride to elute the staining.[226] Furthermore, it produces more reliable results especially in weakly stained monolayers.[226]

In our current study on the ASCs, no mineralised nodules were observed at 14 days of differentiation (Figure 4.5). Darker red stained areas were found in the VC, VC+OM, 80 µM and 100 µM PLA at 14 days. Morphological differences were observed at 14 days where in the VC, cells were thinner and spindle shaped. The VC+OM and PLA exposed cells were larger and less spindle shaped. At 21 days of differentiation, no mineralised nodules were observed, however, darker stained areas were visible at 40-100 µM PLA (Figure 4.6). Morphological differences were seen at 21 days of differentiation between the VC and OM treated cells. Cells were smaller and more spindle shaped in the VC whereas in OM and PLA exposed cells, the cells appeared more round and larger in size.

A study by Li *et al*., compared the bone forming ability of osteogenic differentiated ASCs and compared it to undifferentiated ASCs.[242] They found that after osteogenic differentiation, ASCs showed morphological changes after 14 days, with the presence of mineralised nodules.[242] ASCs became cubic shaped, aggregated and secreted mineralised materials to form nodules.[242] In our current study, morphological changes were visible at 14 and 21 days and the dark areas could possibly be ECM secreted by osteoblasts before nodule formation takes place but no nodules were formed yet.

The dye was eluted and measured spectrophotometrically. Results at 14 days showed the absorbance was not significantly increased in PLA exposed cells compared to the VC+OM (Figure 4.7), however, a slight increase in the absorbance was observed at 80 µM PLA compared to the VC+OM. At 21 days, the absorbance was significantly increased at 80 µM PLA compared to the VC+OM where darker stained areas were observed (Figure 4.7).

A study by Weinzierl *et al*., showed that calcium deposition increased from the 3rd week up to the 5th week of differentiation in human ASCs where the increase in mineralisation was accompanied by an increase in OCN expression.[237] Di Battista *et al*., investigated the behaviour of human ASCs *in vitro* and their capacity to differentiate into osteoblast lineages.[243] Their study found that the ability of ASCs to differentiate into osteoblasts were reduced as the passage number increased.[243] ASCs begin to lose their potential to differentiate into osteoblasts at passage 2 and completely lose the ability altogether at passage 4.[243] Studies conducted on ASCs have shown inconsistent results as some reports state that the age of the donor is important as it can influence the doubling time and gene and protein expression. These factors could have influenced the results obtained in our study.[244]

In the current study on the MG-63 osteosarcoma cells, results indicated that Alizarin Red S staining was not significantly increased at 14 days (Figure 4.8) and 21 days (Figure 4.9) of differentiation after PLA exposure compared to the VC+OM. There were morphological differences evident. In the VC at both 14 and 21 days, cells were irregular in shape and the border between each cell was still distinguishable. The cells treated with OM at 14 and 21 days appeared more round as well as larger and cells were indistinguishable from one another as the borders between each cell was not visible. The cells were closer to one another thus forming a monolayer. The dye was eluted and results at 14 days showed no significant increases in the absorbance compared to the VC+OM. At 21 days, a trend at 20-80 µM PLA showed an increase in absorbance compared to the VC+OM. There was an increase in the absorbance at 21 days compared to 14 days but these increases were not significant (Figure 4.10).

Studies on MG-63 osteosarcoma cells have shown that these cells may be blocked in a pre-osteoblast state. Reports regarding calcium mineralisation have also been inconsistent.[244] Czekanska *et al*., reported that MG-63 cells form a cell layer after 14

days of differentiation with no calcium deposition in the ECM.[238] Thus it was concluded that this cell line is unable to mineralise which corresponds with our present study, as no mineralised nodules were observed, and the cells formed a monolayer from 14 days of differentiation.[238] Wang *et al*., assessed the calcium mineralisation in MG-63 cells at 5 and 7 days and found that calcium content was the highest at 7 days[245], however the study does not mention whether calcium nodules were formed.

Gene expression

Gene expression for early osteoblast-specific markers were analysed. These markers include ALP, RUNX2, Osx, OCN, OPG and RANKL. The gene expression was evaluated relative to GAPDH which was used as the housekeeping gene. In this study, we only used a concentration of 100 µM PLA for gene expression analyses as using a range of concentrations would be costly and more time consuming. As previously mentioned, ALP is a biochemical marker for osteoblast activity thus gene expression of this enzyme was determined.[235] RUNX2 is an important transcription factor in osteoblast differentiation as it is known to be involved in multiple signalling pathways which are activated during osteoblastogenesis.[91] Osx is a transcription factor found downstream of the Wnt signalling pathway. It is known to be an important factor in MSC commitment and osteoblastogenesis.[69] OCN is an ECM protein and a marker of mature osteoblasts.[101] OPG, secreted by osteoblasts, is a decoy receptor for RANKL which blocks RANK-RANKL interaction.[9] A high OPG/RANKL ratio is known to have bone protective effects thus the gene expression of OPG and RANKL was determined in order to examine the OPG/RANKL ratio.[13] Genes expressed by pre-osteoblasts and osteoblasts are shown in Figure 5.1.[246]

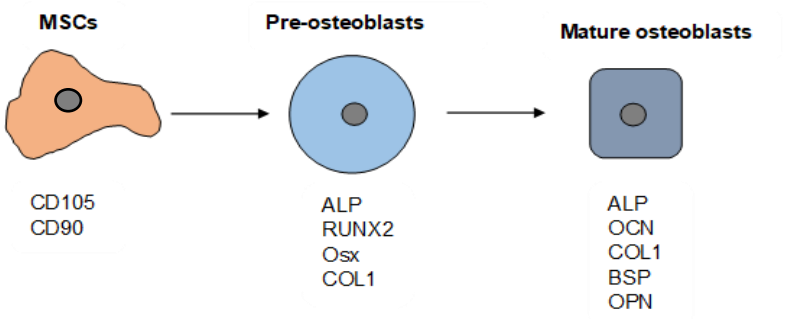

Figure 5.1. Osteogenic markers present during osteoblastogenesis.
Osteoblast-specific markers are expressed at different stages of osteoblast differentiation from MSCs. Early osteogenic markers include ALP, RUNX2, OSX and COL1 while late osteogenic markers include ALP, OCN, COL1, BSP and OPN. Image created from information by Miron *et al*.[246]

Comparisons drawn between the VC and the VC+OM in the ASCs at 7 days of differentiation showed that ALP expression was significantly decreased in the VC+OM compared to the VC which suggests the presence of OM may inhibit ALP expression in this experiment (Figure 4.11A). RUNX2 expression showed an increase in the VC+OM compared to the VC but this was not significant (Figure 4.11B). For Osx, RANKL and the OPG/RANKL ratio gene expression was decreased in the VC+OM compared to the VC (Figure 4.11C, F and G). OCN gene expression showed a minor increase in the VC+OM compared to the VC (Figure 4.11D). These trends were not statistically significant. OPG showed a significant decrease in the VC+OM compared to the VC thus the presence of OM may have inhibited the production of OPG (Figure 4.11E) in this experiment.

In the ASCs at 7 days of differentiation, ALP expression was significantly increased after PLA exposure compared to the VC+OM (Figure 4.11A). RUNX2 expression showed an increase after PLA exposure while Osx and OCN expression showed a minor increase after PLA exposure compared to the VC+OM but this was not significant (Figure 4.11B, C and D). OPG showed a minor decrease in the gene expression after PLA exposure which was not statistically significant, however, RANKL gene expression was significantly decreased after PLA exposure compared to the VC+OM (Figure 4.11E and F). The OPG/RANKL ratio showed a trend that increased after PLA exposure compared to the VC+OM but this was also not significant (Figure 4.11G). This may indicate that osteoblast differentiation was possibly stimulated by

PLA in the ASCs as a high OPG/RANKL ratio indicates bone protective effects were exerted by PLA.

In the ASCs, comparisons were drawn between the VC and the VC+OM at 14 days of differentiation. Increases in the gene expression of ALP, RUNX2, Osx and OPG/RANKL ratio were observed in the VC+OM compared to VC (Figure 4.12A, B, C and G). These changes were not statistically significant. OCN, OPG and RANKL, on the other hand, showed trends where gene expression decreased in the VC+OM compared to the VC (Figure 4.12D, E and F). The changes were not statistically significant. Pre-osteoblasts may not have been present in the VC where cells were grown in the absence of OM thus we would have expected a lower osteoblast-specific gene expression and a low OPG/RANKL ratio in these cells.

At 14 days of differentiation in the ASCs, ALP gene expression was significantly increased after PLA exposure compared to the VC+OM. Trends in RUNX2, Osx, OCN, OPG, RANKL and the OPG/RANKL ratio showed increases in gene expression after PLA exposure compared to the VC+OM, however, these increases were not statistically significant. These results show that osteoblast differentiation may have been stimulated by PLA along with the activation of osteoblast signalling pathways, however, more work is required to confirm this.

In a study by Liu *et al*., cryopreserved and non-cryopreserved human ASCs expressed ALP, COL1 and OPN in osteoblast induced ASCs (ASCs exposed to OM) and non-induced ASCs (ASCs grown in the absence of OM) after 14 days of plating as determined by PCR.[247] In non-induced ASCs, OCN was not expressed.[247] This could explain the detectable levels of ALP expressed in the VC at 7 days and 14 days in our study. In the study by Liu *et al*., gene expression was only determined at 14 days of differentiation.

Egusa, *et al*., investigated the expression of ECM-related genes in ASCs and bone marrow MSCs.[248] Undifferentiated ASCs and bone marrow MSCs highly expressed osteoblast-specific genes that represent several phenotypes.[248] Osteogenic differentiated ASCs demonstrated almost no significant upregulation in osteogenic markers and rather a downregulation in ECM-related genes were observed.[248] The study concluded that small changes in mRNA expression may cause major phenotypic

effects and osteogenic differentiation of ASCs and bone marrow MSCs may supress ECM gene expression.[248] This may explain the lack of upregulated osteoblast-specific genes observed in our study in the ASCs at 7 and 14 days after exposure to PLA and the upregulated genes in the undifferentiated ASCs (VC) compared to the VC+OM.

In the MG-6 cells at 7 and 14 days, the gene expression of ALP, RUNX2 and OCN was significantly increased in the VC+OM compared to the VC (Figure 4.13A, B and D and Figure 4.14A, B and D). Osx was increased at 7 days, but not significantly, in the VC+OM compared to the VC (Figure 4.13C) while at 14 days, Osx was significantly reduced in the VC+OM compared to the VC (Figure 4.14C). Osx is an early marker found downstream of the Wnt signalling pathway[90] thus osteoblastogenesis may have been stimulated through RUNX2 activation independent of the Wnt signalling pathway. OPG was reduced, but not significantly at 7 and 14 days in the VC+OM compared to the VC (Figure 4.13E and Figure 4.14E). RANKL expression was significantly increased at 7 and 14 days in the VC+OM compared to the VC (Figure 4.13F and Figure 4.14F). The OPG/RANKL ratio was significantly reduced in the VC+OM compared to the VC at 7 and 14 days (Figure 4.13G and Figure 4.14G) which indicates that the presence of OM may exert bone protective effects through osteoblasto-genesis.

ALP expression was increased, but not significantly, at 7 days after PLA exposure compared to the VC+OM (Figure 4.13A), however, was significantly reduced at 14 days after PLA exposure compared to the VC+OM (Figure 4.14A). RUNX2 and OCN were significantly reduced at both 7 and 14 days after exposure to PLA compared to the VC+OM (Figure 4.13B and D and Figure 4.14 B and D). Expression of Osx at 7 days was increased, but not significantly, after PLA exposure compared to VC+OM (Figure 4.13C) while at 14 days, Osx was significantly decreased after exposure to PLA compared to VC+OM (Figure 4.14C). The significant reductions in these markers may indicate that osteoblast formation was not stimulated after PLA exposure in the MG-63 cells. Gene expression of OPG was reduced, but not significantly (Figure 4.13E and Figure 4.14E), at 7 and 14 days while RANKL was significantly reduced at both 7 and 14 days after exposure to PLA compared to the VC+OM (Figure 4.13F and Figure 4.14F). The OPG/RANKL ratio was increased, but not significantly at both 7 and 14 days after PLA exposure compared to VC+OM (Figure 4.13G and Figure

4.14G). The increased OPG/RANKL ratio at both 7 and 14 days may be indicative of osteoblastogenesis, however, the increase was not statistically significant thus more work is required to confirm this.

Kasonga *et al*., determined whether free fatty acid receptor 4 had any effects on UFA osteoblast-like cells. MC3T3-E1 murine pre-osteoblasts were transfected to silence the expression of free fatty acid receptor 4, β-arrestin 2 or $G_{\alpha q}$ and were exposed to OM and 100 μM PLA for 7-14 days.[9] Results indicated that 100 μM PLA increased OPG gene expression in control and $G_{\alpha q}$ negative cells.[9] PLA did not affect the OPG gene expression in free fatty acid receptor 4 and β-arrestin 2 silenced cells.[9] RANKL gene expression was not affected by PLA in control plasmid or in the free fatty acid receptor 4, β-arrestin 2 and $G_{\alpha q}$ negative cells.[9] Our results contradict those of Kasonga *et al*., who showed PLA (100 μM) had no effect on RANKL expression, while in our study, RANKL expression was reduced. It is important to note that Kasonga *et al*., made use of MC3T3-E1 murine cells while in our study, we used ASCs and MG-63 cells.

ASCs may differentiate into osteoblasts more readily than MG-63 cells. Gene expression at 21 days could not be determined in these cells due to technical constraints. Gene expression for late osteoblast-specific markers and PPAR-γ for adipogenesis could therefore not be evaluated. A summary of osteoblast-specific genes expressed at different stages of osteoblast differentiation can be found in Figure 5.1.[247] In our study, several pre-osteoblast genes were upregulated in the ASCs after PLA treatment although not significantly, while in the MG-63 cells, osteoblast-specific gene expression was mostly reduced indicating that osteoblast formation may have been supressed by PLA in MG-63 cells. The addition of OM exerts mostly a stimulatory effect on MG-63 cells but this effect is lost after the addition of PLA. MG-63 cells may be arrested in a pre-osteoblast state.[244]

Protein expression

In this study, western blotting was used to detect and analyse protein expression. The relative protein expression of pERK/ERK was determined at 3, 6 and 24 hours after 100 μM PLA exposure in the presence of OM. In MG-63 cells, results indicated that relative pERK/ERK expression was not significantly changed at 3, 6 and 24 hours (Figure 4.15) which was confirmed after densitometry analysis using ImageJ software

(Figure 4.16A, B and C). These short incubation times measure the activation of the ERK pathway as cell signalling is stimulated early on in differentiation. At 3 and 6 hours, trends showed that pERK/ERK expression was increased after PLA exposure compared to the VC+OM, however, these were not significant. At 24 hours pERK/ERK expression was reduced after PLA exposure compared to the VC+OM, however, this was again not significant. Due to technical constraints, the relative gene expression for pJNK/JNK and pp38/p38 could not be determined. This would have given a more holistic view on the effects of PLA on the MAPK pathway. MAPK are important in the regulation of osteogenic differentiation in MSCs as ERK is activated during differentiation. ERK inhibition has been known to reduce bone mineralisation while pERK expression regulates RUNX2 and Osx expression.[249] The increased pERK/ERK expression at 3 and 6 hours may indicate that osteogenesis was stimulated through the MAPK pathway, however, it was not significant. The reduction in pERK/ERK expression may have caused the reduction in osteoblast gene expression in MG-63 cells as the ERK MAPK pathway is upstream from osteoblast gene regulation.[250]

Hu *et al*., conducted a study on MG-63 cells where cells were treated with cadmium for 24 or 48 hours.[251] Cytotoxic doses of cadmium were shown to induce apoptosis in the MG-63 osteosarcoma cells.[251] Protein expression of pp38 MAPK was induced while pERK1/2 was inhibited which led to apoptosis. The study proposed that decreased pp38 inhibited apoptosis caused by cadmium.[251] In the current study, pERK1/2 was upregulated at 6 hours but not significantly, indicating PLA may have a stimulating effect on MAPK pathways during osteoblast formation. More work is however required to confirm this.

Adipogenesis

Oil Red O staining is a histochemical staining used to detect lipid vacuoles within adipocytes by staining triglycerides present in the cytoplasm.[177] In the current study, results revealed no significant increases in the presence of lipid droplets at 21 days when compared to the VC+OM in the ASCs (Figure 4.17). Morphological changes were seen between the VC and cells exposed to OM (VC+OM). In the VC, cells were triangular shaped with long thing fibres branching out to neighbouring cells. In the VC+OM, ASCs were more round than triangular with almost no fibres protruding from the cells. As the concentration of PLA increased, ASCs became larger with more fibres

branching out to neighbouring cells. The ASCs also formed a monolayer as the PLA concentration increased to 100 μM. In the positive control, lipid accumulation can be seen in the lipid droplets stained red which indicates the cells may have been adipocytes with lipid droplets stored in the vacuoles. In the ASCs, after elution of the dye, a slight increase in absorbance was seen in cells treated with 20-100 μM PLA compared to the VC+OM, however, this was not statistically significant (Figure 4.18). Osteoblastic cells have been shown to undergo transdifferentiation into adipocytes when treated with LCPUFA which could explain the increases seen after PLA exposure.[140]

Results revealed no significant increases in the presence of lipid droplets after PLA exposure when compared to the VC+OM in MG-63 cells (Figure 4.19). Morphological changes were seen in the VC compared to the VC+OM and cells exposed to PLA. In the VC, MG-63 cells were irregular shaped with some dark stained areas. In the VC+OM and PLA treated cells, the cell border was more visible with some round cells present in between elongated irregular shaped cells. Darker stained areas could be seen at 60 μM and 100 μM PLA compared to the VC+OM. At 80 μM and 100 μM PLA, the red dye could be seen within several cells which indicates lipid droplets may have been present within cells at these high concentrations. The graph showed that the absorbance was increased from 20-100 μM PLA which was significant at 100 μM PLA (Figure 4.20). MG-63 cells highly express PPAR-γ[140] which can be activated by LCPUFA[252] and possibly PLA thus causing lipid accumulation to occur at a high PLA concentration.

Our study is in agreement with results published by Coetzee *et al*., where MC3T3-E1 murine pre-osteoblasts were exposed to PUFA, DHA and AA.[177] Oil Red O staining demonstrated that the PUFA did not induce lipid-filled vacuoles in the cells thus adipogenesis may not have been stimulated.[177] In the study, OA was used as a positive control to induce adipogenesis.[177] In our results, Oil Red O stained lipid deposits cannot clearly be seen thus, adipocytes may not have formed, however, a high concentration of PLA may have caused an increase in lipid accumulation. The presence of adipocytes will only be confirmed after analyses of adipogenesis-specific markers in a later study.

Durandt *et al*., used flow cytometry to detect adipocyte subpopulations as technical difficulties are often encountered during Oil Red O staining.[121] The study mentioned that a fluorescent lipophilic stain, 9-diethylamino-2-hydroxy-5H-benzo (α) phenoxazine-5-one (NR) could be used, or alternatively 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BDP) which has been reported to be more sensitive than NR in detecting intracellular lipid droplets.[121]

Aldridge *et al*., conducted a study on four methods which could be used to assess adipocytes. Flow cytometry using NR was shown to be less subjective and more quantitative than Oil Red O staining.[231] Lansdown *et al*., showed that flow cytometry using NR or qPCR is recommended to evaluate adipogenesis.[253] Durandt *et al*., concluded that BDP is more sensitive in detecting intracellular lipids, however, the unique fluorescence emission spectrum of NR, allows for the identification of mature adipocytes.[121] In this study, adipocyte-specific gene expression could be determined along with osteoblast-specific gene expression to determine whether adipogenesis was induced or not. PPAR-γ is a marker of adipogenesis and was originally included in our study of gene expression, however, due to technical constraints, this could not be completed.

5.2 Integration with current knowledge

FA can be measured in the plasma, erythrocytes and adipose tissue.[254] Lipid profiles from adipose tissue, reflects FA intake over several years while lipid profiles from erythrocytes reflects FA intake over several months. Plasma lipid profiles reflects FA intake over several weeks thus is more suitable to reflect the present dietary behaviours.[254] In a study by Abdelmagid *et al*., the average concentrations of several FA present in total plasma in ethno-culturally diverse, young Canadian men and women between the ages of 20-29 were determined.[254] The plasma lipid profiles were found to contain OA, which had a minimum concentration of 178.7 μM and a maximum concentration of 3 210.5 μM with an average concentration of 1 285.5 μM.[254] *Cis*9 PLA was found to have a minimum concentration of 27.7 μM and a maximum concentration of 555.9 μM with an average concentration of 133.0 μM.[254] *Trans*9 PLA ranged from trace amounts to a maximum concentration of 65.2 μM with an average concentration of 17.0 μM.[254] The highest concentration used in our study was 100 μM thus the concentration used in the gene and protein expression experiments are within the

physiological ranges found in the study mentioned above. However, the plasma total lipids also includes total cholesterol, triglycerides and free fatty acids found in the blood[254] thus the amount of FA obtained from the diet may be lower than observed in the study.

Current studies have shown that some UFA such as DHA, EPA and PLA exert bone protective effects.[9, 12, 175, 255] A study conducted on murine osteoclast and osteoblast cells *in vitro* showed that UFA such as DHA, EPA, PLA and OA promote the gene expression of MC3T3-E1 murine pre-osteoblasts though free fatty acid receptor 4.[9] Furthermore, DHA, EPA, PLA and OA upregulated OPG gene expression while RANKL remained the same thus increasing the OPG/RANKL ratio.[9]

Casado-Diaz *et al*., used human MSCs isolated from bone marrow and demonstrated that AA inhibited the expression of osteoblast-specific genes in human MSCs derived from bone marrow such as RUNX2, Osx and ALP at 20 μM at 7 days of differentiation.[175] DHA and EPA (40 μM) inhibited the expression of RUNX2 and Osx and not ALP.[175] At 14 days of differentiation, EPA and AA (20 μM) both inhibited the gene expression of RUNX2, however, DHA stimulated the gene expression of ALP at 20 μM.[175] Results from the study indicated that AA induces osteoclastogenesis and bone resorption more readily than EPA and DHA.[175]

Viegas *et al*., conducted *in vitro* research on AA, EPA and DHA on VSa14 cells, which are skeletal cells derived from the vertebra of gilthead seabream.[255] EPA and AA was found to inhibit ECM mineralisation while DHA was shown to stimulate mineralisation.[255] OPN gene expression was significantly reduced by EPA and AA but remained unchanged after DHA exposure.[255] The study concluded that the effects of PUFA are specific to each molecule, process and target gene.[255] PLA may exert different effects on osteoblasts depending on the target gene and receptor that is activated thus some osteoblast genes may be downregulated while other osteoblast genes are upregulated.

There are currently no studies published on the effects of PLA, an omega-7 MUFA, on osteoblastogenesis in human ASCs and MG-63 osteosarcoma cell lines. Minetti *et al*., conducted a study on human osteoblasts where the effects of brown seaweed, *Padina pavonica*, was investigated.[256] Acetonic extract of P. *pavonica* (EPP) was chemically

characterised and found to contain 26.7% MUFA of which 6.29% is PLA.[256] EPP increased the expression of early and late differentiation markers such as ALP, OCN and OPG thus promoting an osteoblastic phenotype.[256]

PLA has been used in a previous study to determine its effects on RANKL-induced osteoclastogenesis. Van Heerden *et al*., conducted a study on RAW264.7 murine macrophages and found that PLA reduced the number and size of tartrate-resistant acid phosphatase positive osteoclasts which coincided with a decrease in the expression of bone resorption markers.[12] PLA also inhibited the activation of ERK and JNK.[12] The exact mechanism of action of PLA has yet to be determined in human osteoblasts as previous studies by Kasonga *et al*., showed that free fatty acid receptor 4 is required for PLA to exert an effect on RAW264.7 murine macrophages and murine MC3T3-E1 cells through the free fatty acid receptor 4/β-arrestin signalling pathway. The study indicated that free fatty acid receptor 4 activation inhibits osteoclastogenesis through inhibiting MAPK signalling pathways.

Primary human cells are relevant in clinical studies, however, the cell phenotype may be dependent on donor-specific factors with long procedures for isolation and limited accessibility.[244] SaOS-2 cells secrete a mineralised matrix and have an expression profile similar to human osteoblasts but are sensitive to inorganic phosphates and may not be representative of all osteoblast phenotypes.[244] MG-63 cells respond to hormones and express human integrin profiles in a similar manner to human osteoblasts, however, they are arrested in a pre-osteoblast state and conflicting reports regarding mineralisation have been published.[244] In our study, the stage of the cells during osteogenic differentiation may influence the gene or protein expression. ASCs are human stromal cells which only have a short usage time while MG-63 cells seems to be arrested in a pre-osteoblast state thus have different responses to osteoblast differentiation. The exposure of PLA could be more beneficial to MSCs than cells in a pre-osteoblast state such as MG-63 cells as it may have downregulated protein and osteoblast-specific gene expression in pre-osteoblasts and the cells may lack the potential for mineralisation.

Chapter 6

Conclusion

The study investigated for the first time, the effects of PLA, an omega-7 MUFA, on human ASCs and MG-63 osteosarcoma cell lines. The study investigated the effects of PLA on cell viability, ALP activity, calcium mineralisation, gene expression and protein expression. This may expand our knowledge of how PLA can influence the bone remodelling cycle and whether it may affect the MAPK signalling mechanisms during osteoblast formation in human cells.

In both cells lines, PLA had no effects on cell viability at the tested concentrations. In ASCs, the increase of ALP activity at 7 days and the increase in Alizarin Red S staining at 21 days indicates that PLA exerts an effect on osteoblast differentiation though the results were not significant. ALP gene expression was significantly increased at 7 days which coincides with results seen in the ALP activity after PLA exposure. ALP activity was still increased at 14 days after PLA exposure and this may indicate that the ASCs were differentiating into osteoblast-like cells. The significant reduction in RANKL gene expression at 7 days compared to 14 and 21 days after PLA exposure may have been due to the cells being early osteoblasts. Adipogenesis could not be confirmed as no significant increases in the presence of lipid vacuoles were observed and gene expression would need to be determined to confirm whether adipogenesis was induced in these cells.

In the MG-63 cells, increases in ALP activity and mineralisation at 21 days indicates that PLA exerted an effect on osteoblast differentiation though the results were not significant. The significant decreases of osteoblast-specific gene expression after PLA exposure contradicts the findings demonstrated by ALP activity assay and Alizarin Red S staining but this may be due to the MG-63 cells being arrested in a pre-osteoblastic state thus the cells cannot fully differentiate into terminal osteoblasts. Gene expression needs to be determined for 21 days of differentiation to confirm this. The increase in relative pERK/ERK expression at 3 and 6 hours after PLA exposure might indicate that the MAPK pathway was activated in response to PLA, however the increase was

not significant. The presence of lipid vacuoles was significantly increased at the highest concentration of PLA as it may have activated the PPAR-γ receptors and stimulated the intake of the FA into lipid vacuoles but we cannot confirm the presence of adipocytes until adipogenesis-specific gene expression has been determined.

To conclude, PLA showed some changes in osteoblast differentiation and the MAPK signalling pathway and trends may infer that osteoblast-like cells were formed after PLA exposure, particularly in the ASCs, although more experiments need to be conducted to confirm this.

6.1 Limitations of the study

During the study, a number of limitations were experienced. The current study may provide essential information when designing future studies based on this study. ALP activity assay measures the amount of ALP released from cells while qPCR measures the gene expression of ALP. Future studies could include an ALP activity staining to assess the expression of ALP as it is normalised to total protein content.

Alizarin Red S staining is used to detect calcium mineralisation by osteoblasts. A positive control could be used to ensure that the staining effectively stains calcium. It would provide a means for accurate comparison.

Expression of the genes mentioned in this study could also be determined at 21 days of differentiation or beyond 21 days to determine whether the genes are expressed at a later stage of differentiation. Gene expression could also be determined for late osteoblast-specific markers and adipogenesis markers such as PPAR-γ as originally planned in the study, but could not be carried out.

ASCs derived from lipoaspirates have a short window for use as an osteoblast model. Weinzierl *et al*., determined that ASCs lose their osteoblast potential after the third passage, however, Di Battista *et al*., determined that ASCs already start to lose their osteogenic potential after passage two and its potential is completely lost at passage five.[237, 243] ASCs may not be a suitable model for long-term osteoblastic studies which may require culturing cells beyond 21 days.

6.2 Recommendations for further studies

A substantial amount of research has been conducted on the benefits of PUFA on health, although some aspects still need to be explored. With the aim of understanding the link between the health benefits of MUFA and bone, a potential treatment could be discovered to alleviate treatments involving costly drug-dependence.

The use of other cells lines such as human foetal osteoblasts (hFOB 1.19), bone marrow MSCs or SaOS-2 cells could be used as alternative models for cell culture. Evaluating the gene expression of other osteoblast-specific genes such as COL1, BSP and OPN can be included.

The present study could form the foundation to continue research on MUFA and specifically PLA in bone cells. Future research could include *in vivo* work using PLA on murine models to observe the effects of PLA on osteoblasts and the bone remodelling cycle.

Due to contradictory findings between the ASCs and MG-63 cells, the study cannot conclude whether PLA has a beneficial effect on bone. In the ASCs, it stimulated osteoblast gene expression while in the MG-63 cells it inhibited osteoblast gene expression. Van Heerden *et al*., also demonstrated that PLA exerted inhibitory effects on osteoclast formation[12] but this may not affect osteoblast formation.

References

1. Sims NA, Gooi JH, editors. Bone remodeling: Multiple cellular interactions required for coupling of bone formation and resorption. Semin Cell Dev Biol; 2008: Elsevier.
2. Rosen CJ, Bouxsein ML. Mechanisms of disease: is osteoporosis the obesity of bone? Nat Rev Rheumatol. 2006;2(1):35.
3. International Osteoporosis Foundation. [Internet]. Broken Bones, Broken Lives: A roadmap to solve the fragility fracture crisis in the United Kingdom: IOF; [cited 2017]. Available from: https://www.iofbonehealth.org/broken-bones-broken-lives.
4. Hu L, Yin C, Zhao F, Ali A, Ma J, Qian A. Mesenchymal stem cells: cell fate decision to osteoblast or adipocyte and application in osteoporosis treatment. Int J Mol Sci. 2018;19(2):360.
5. Garg P, Mazur MM, Buck AC, Wandtke ME, Liu J, Ebraheim NA. Prospective review of mesenchymal stem cells differentiation into osteoblasts. Orthop Surg. 2017.
6. van Vollenstee FA, Dessels C, Kallmeyer K, de Villiers D, Potgieter M, Durandt C, et al. Isolation and characterization of adipose-derived stromal cells. Stem Cell Processing: Springer; 2016. p. 131-61.
7. Moro C, Capel F. Regulation of Skeletal Muscle Metabolism by Saturated and Monounsaturated Fatty Acids. Nutrition and Skeletal Muscle: Elsevier; 2019. p. 347-58.
8. Watkins BA, Li Y, Lippman HE, Feng S. Modulatory effect of omega-3 polyunsaturated fatty acids on osteoblast function and bone metabolism. Prostaglandins Leukot Essent Fatty Acids. 2003;68(6):387-98.
9. Kasonga AE, Kruger MC, Coetzee M. Free fatty acid receptor 4-β-arrestin 2 pathway mediates the effects of different classes of unsaturated fatty acids in osteoclasts and osteoblasts. Biochim Biophys Acta Mol Cell Biol Lipids. 2019;1864(3):281-9.
10. Kruger MC, Coetzee M, Haag M, Weiler H. Long-chain polyunsaturated fatty acids: selected mechanisms of action on bone. Prog Lipid Res. 2010;49(4):438-49.
11. Gillingham LG, Harris-Janz S, Jones PJ. Dietary monounsaturated fatty acids are protective against metabolic syndrome and cardiovascular disease risk factors. Lipids. 2011;46(3):209-28.
12. van Heerden B, Kasonga A, Kruger MC, Coetzee M. Palmitoleic Acid Inhibits RANKL-Induced Osteoclastogenesis and Bone Resorption by Suppressing NF-κB and MAPK Signalling Pathways. Nutrients. 2017;9(5):441.
13. Clarke B. Normal bone anatomy and physiology. Clin J Am Soc Nephrol. 2008;3(Supplement 3):S131-S9.
14. Pino AM, Rodríguez JP. Is fatty acid composition of human bone marrow significant to bone health? Bone. 2019;118:53-61.
15. Weiner S, Wagner HD. The material bone: structure-mechanical function relations. Annu Rev Mater Res. 1998;28(1):271-98.
16. Ferraiolo JA, Dana JD, Dana ES. A systematic classification of nonsilicate minerals. Bulletin of the AMNH; v. 172, article 1. 1982.
17. Katz EP, Li S-T. Structure and function of bone collagen fibrils. J Mol Biol. 1973;80(1):1-15.

18. Currey J. Physical characteristics affecting the tensile failure properties of compact bone. J Biomech. 1990;23(8):837-44.
19. Bourne GH. The biochemistry and physiology of bone: Elsevier; 2014.
20. Birk DE, Silver FH, Trelstad RL. Matrix assembly. Cell biology of extracellular matrix: Springer; 1991. p. 221-54.
21. Robinson RA. An electron-microscopic study of the crystalline inorganic component of bone and its relationship to the organic matrix. JBJS. 1952;34(2):389-476.
22. Lowenstam H. Minerals and macromolecules, Biomineralization Processes. On biomineralization. 1989;7:25-49.
23. Weiner S, Price PA. Disaggregation of bone into crystals. Calcif Tissue Int. 1986;39(6):365-75.
24. Elliott S, Robinson R. The water content of bone. I. The mass of water, inorganic crystals, organic matrix, and CO2 space components in a unit volume of the dog bone. J Bone Joint Surg Am. 1957;39(1):167-88.
25. Tortora GJ. Principles of human anatomy: Wiley; 2004.
26. Gunnarsson CA, Sanborn B, Foster M, Moy P, Weerasooriya T. Initiation fracture toughness of human cortical bone as a function of loading rate. Dynamic Behavior of Materials, Volume 1: Springer; 2013. p. 45-56.
27. Rho J-Y, Kuhn-Spearing L, Zioupos P. Mechanical properties and the hierarchical structure of bone. Med Eng Phys. 1998;20(2):92-102.
28. Morgan EF, Barnes GL, Einhorn TA. The bone organ system: form and function. Osteoporosis: Elsevier; 2013. p. 3-20.
29. Markings B. The skeletal system. 1995.
30. Nather A, Ong H, Aziz Z. Structure of bone. Bone grafts and bone substitutes: basic science and clinical applications: World Scientific; 2005. p. 3-17.
31. Ohlsson C, Sjögren K. Effects of the gut microbiota on bone mass. Trends Endocrinol Metab. 2015;26(2):69-74.
32. Nicholls JJ, Brassill MJ, Williams GR, Bassett JD. The skeletal consequences of thyrotoxicosis. J Endocrinol. 2012;213(3):209-21.
33. Everts V, Delaisse J, Korper W, Jansen D, Tigchelaar-Gutter W, Saftig P, et al. The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation. J Bone Miner Res. 2002;17(1):77-90.
34. Dougall WC, Glaccum M, Charrier K, Rohrbach K, Brasel K, De Smedt T, et al. RANK is essential for osteoclast and lymph node development. Genes Dev. 1999;13(18):2412-24.
35. Leibbrandt A, Penninger JM. RANK/RANKL: regulators of immune responses and bone physiology. Ann N Y Acad Sci. 2008;1143(1):123-50.
36. Rossi M, Battafarano G, Barbuti D, Del Fattore A. Osteopetrosis. Orthopedic Surgery Clerkship: Springer; 2017. p. 757-61.
37. Vallet M, Ralston SH. Biology and treatment of Paget's disease of bone. J Cell Biochem. 2016;117(2):289-99.
38. Simonet W, Lacey D, Dunstan C, Kelley M, Chang M-S, Lüthy R, et al. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell. 1997;89(2):309-19.
39. Tsuda E, Goto M, Mochizuki S-i, Yano K, Kobayashi F, Morinaga T, et al. Isolation of a novel cytokine from human fibroblasts that specifically inhibits osteoclastogenesis. Biochem Biophys Res Commun. 1997;234(1):137-42.
40. Schmid C, Schläpfer I, Futo E, Waldvogel M, Schwander J, Zapf J, et al. Triiodothyronine (T3) stimulates insulin-like growth factor (IGF)-1 and IGF

binding protein (IGFBP)-2 production by rat osteoblasts in vitro. Acta Endocrinol (Copenh). 1992;126(5):467-73.
41. Rodan GA. Introduction to bone biology. Bone. 1992(13(Suppl. 1)):S3–6.
42. Centrella M, McCarthy T, Canalis E. Transforming growth factor-beta and remodeling of bone. JBJS. 1991;73(9):1418-28.
43. Harris SE, Harris MA, Mahy P, Feng JQ, Mundy GR, Wozney J. Expression of bone morphogenetic protein messenger RNAs by normal rat and human prostate and prostate cancer cells. Prostate. 1994;24(4):204-11.
44. Vukicevic S, Latin V, Chen P, Batorsky R, Reddi A, Sampath TK. Localization of osteogenic protein-1 (bone morphogenetic protein-7) during human embryonic development: high affinity binding to basement membranes. Biochem Biophys Res Commun. 1994;198(2):693-700.
45. Vukicevic S, Paralkar VM, Cunningham NS, Gutkind JS, Reddi A. Autoradiographic localization of osteogenin binding sites in cartilage and bone during rat embryonic development. Dev Biol. 1990;140(1):209-14.
46. Rydziel S, Ladd C, McCarthy TL, Centrella M, Canalis E. Determination and expression of platelet-derived growth factor-AA in bone cell cultures. Endocrinol. 1992;130(4):1916-22.
47. Hu Y, Chan E, Wang SX, Li B. Activation of p38 mitogen-activated protein kinase is required for osteoblast differentiation. Endocrinol. 2003;144(5):2068-74.
48. Klein-Nulend J, Nijweide PJ, Burger EH. Osteocyte and bone structure. Curr Osteoporos Rep. 2003;1(1):5-10.
49. Zanatta LC, Boguszewski CL, Borba VZ, Kulak CA. Osteocalcin, energy and glucose metabolism. Arq Bras Endocrinol Metabol. 2014;58(5):444-51.
50. Sodek J, Ganss B, McKee M. Osteopontin. Crit Rev Oral Biol Medicine. 2000;11(3):279-303.
51. Delany A, Amling M, Priemel M, Howe C, Baron R, Canalis E. Osteopenia and decreased bone formation in osteonectin-deficient mice. J Clin Invest. 2000;105(7):915-23.
52. Young MF, Kerr JM, Ibaraki K, Heegaard A-M, Robey PG. Structure, expression, and regulation of the major noncollagenous matrix proteins of bone. Clin Orthop Relat Res. 1992(281):275-94.
53. Lane TF, Sage EH. The biology of SPARC, a protein that modulates cell-matrix interactions. FASEB J. 1994;8(2):163-73.
54. Sims NA. EPHs and ephrins: many pathways to regulate osteoblasts and osteoclasts. IBMS Bonekey. 2010;7(9):304-13.
55. Marcus R, Majumder S. The nature of osteoporosis. Osteoporosis: Elsevier; 2001. p. 3-17.
56. Black DM, Rosen CJ. Postmenopausal osteoporosis. New Engl J Med. 2016;374(3):254-62.
57. Hough T FG, Adib MG, Nauroy L. The Middle East & Africa regional audit: epidemiology, costs & burden of osteoporosis in 2011. Arch Osteoporos. 2011.
58. Drake MT, Clarke BL, Lewiecki EM. The pathophysiology and treatment of osteoporosis. Clin Ther. 2015;37(8):1837-50.
59. Raisz LG. Pathogenesis of osteoporosis: concepts, conflicts, and prospects. J Clin Invest. 2005;115(12):3318-25.
60. Sözen T, Özışık L, Başaran NÇ. An overview and management of osteoporosis. Eur J Rheumatol. 2017;4(1):46.

61. Borrelli J. The Relationship of Peak Bone Mass, Aging, and Bone Loss to Osteoporosis and Fragility Fractures. Arthroplasty for the Treatment of Fractures in the Older Patient: Springer; 2018. p. 3-17.
62. Ridge SM, Sullivan FJ, Glynn SA. Mesenchymal stem cells: key players in cancer progression. Mol Cancer. 2017;16(1):31.
63. Chhabra A, Zijerdi D, Zhang J, Kline A, Balian G, Hurwitz S. BMP-14 deficiency inhibits long bone fracture healing: a biochemical, histologic, and radiographic assessment. J Orthop Trauma. 2005;19(9):629-34.
64. Zhou S, Chen S, Jiang Q, Pei M. Determinants of stem cell lineage differentiation toward chondrogenesis versus adipogenesis. Cell Mol Life Sci. 2019;76(9):1653-80.
65. Schäffler A, Büchler C. Concise review: adipose tissue-derived stromal cells—basic and clinical implications for novel cell-based therapies. Stem Cells. 2007;25(4):818-27.
66. Takada I, Kouzmenko AP, Kato S. Wnt and PPARγ signaling in osteoblastogenesis and adipogenesis. Nat Rev Rheumatol 2009;5(8):442.
67. Bennett CN, Longo KA, Wright WS, Suva LJ, Lane TF, Hankenson KD, et al. Regulation of osteoblastogenesis and bone mass by Wnt10b. Proc Natl Acad Sci. 2005;102(9):3324-9.
68. Felber K, Elks PM, Lecca M, Roehl HH. Expression of osterix is regulated by FGF and Wnt/β-catenin signalling during osteoblast differentiation. PLoS One. 2015;10(12):e0144982.
69. Rutkovskiy A, Stensløkken K-O, Vaage IJ. Osteoblast differentiation at a glance. Med Sci Monit Basic Res. 2016;22:95.
70. Narcisi R, Cleary MA, Sivasubramaniyan K, Brama PA, van Osch GJ. MSC Populations for Cartilage Regeneration. Cartilage: Springer; 2017. p. 35-57.
71. Terzoudis S, Zavos C, Koutroubakis IE. The bone and fat connection in inflammatory bowel diseases. Inflamm Bowel Dis. 2014;20(11):2207-17.
72. Aronow MA, Gerstenfeld LC, Owen TA, Tassinari MS, Stein GS, Lian JB. Factors that promote progressive development of the osteoblast phenotype in cultured fetal rat calvaria cells. J Cell Physiol. 1990;143(2):213-21.
73. Vater C, Kasten P, Stiehler M. Culture media for the differentiation of mesenchymal stromal cells. Acta Biomater. 2011;7(2):463-77.
74. Franceschi RT, Iyer BS. Relationship between collagen synthesis and expression of the osteoblast phenotype in MC3T3-E1 cells. J Bone Miner Res. 1992;7(2):235-46.
75. Xiao G, Gopalakrishnan R, Jiang D, Reith E, Benson MD, Franceschi RT. Bone morphogenetic proteins, extracellular matrix, and mitogen-activated protein kinase signaling pathways are required for osteoblast-specific gene expression and differentiation in MC3T3-E1 cells. J Bone Miner Res. 2002;17(1):101-10.
76. Foster B, Nociti F, Swanson E, Matsa-Dunn D, Berry J, Cupp C, et al. Regulation of cementoblast gene expression by inorganic phosphate in vitro. Calcif Tissue Int. 2006;78(2):103-12.
77. Fatherazi S, Matsa-Dunn D, Foster B, Rutherford R, Somerman M, Presland R. Phosphate regulates osteopontin gene transcription. J Dent Res. 2009;88(1):39-44.
78. Bellows C, Aubin J, Heersche J. Initiation and progression of mineralization of bone nodules formed in vitro: the role of alkaline phosphatase and organic phosphate. Bone Miner. 1991;14(1):27-40.

79. Martland M, Robison R. The possible significance of hexosephosphoric esters in ossification: part VII. The bone phosphatase. Biochem J. 1927;21(3):665.
80. Hunter GK, Goldberg HA. Modulation of crystal formation by bone phosphoproteins: role of glutamic acid-rich sequences in the nucleation of hydroxyapatite by bone sialoprotein. Biochem J. 1994;302(Pt 1):175.
81. Malaval L, Wade-Guéye NM, Boudiffa M, Fei J, Zirngibl R, Chen F, et al. Bone sialoprotein plays a functional role in bone formation and osteoclastogenesis. J Exp Med. 2008;205(5):1145-53.
82. Geoffroy V, Kneissel M, Fournier B, Boyde A, Matthias P. High bone resorption in adult aging transgenic mice overexpressing cbfa1/runx2 in cells of the osteoblastic lineage. Mol Cell Biol. 2002;22(17):6222-33.
83. Conget PA, Minguell JJ. Adenoviral-mediated gene transfer into ex vivo expanded human bone marrow mesenchymal progenitor cells. Exp Hematol. 2000;28(4):382-90.
84. Yang S, Wei D, Wang D, Phimphilai M, Krebsbach PH, Franceschi RT. In vitro and In vivo synergistic interactions between the Runx2/Cbfa1 transcription factor and bone morphogenetic protein-2 in stimulating osteoblast differentiation. J Bone Miner Res. 2003;18(4):705-15.
85. Frendo J-L, Xiao G, Fuchs S, Franceschi RT, Karsenty G, Ducy P. Functional hierarchy between two OSE2 elements in the control of osteocalcin gene expression in vivo. J Biol Chem. 1998;273(46):30509-16.
86. Kern B, Shen J, Starbuck M, Karsenty G. Cbfa1 contributes to the osteoblast-specific expression of type I collagen genes. J Biol Chem. 2001;276(10):7101-7.
87. Komori T. Regulation of osteoblast differentiation by transcription factors. J Cell Biochem. 2006;99(5):1233-9.
88. Ding J, Ghali O, Lencel P, Broux O, Chauveau C, Devedjian J, et al. TNF-α and IL-1β inhibit RUNX2 and collagen expression but increase alkaline phosphatase activity and mineralization in human mesenchymal stem cells. Life Sci. 2009;84(15-16):499-504.
89. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, et al. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell. 2002;108(1):17-29.
90. Baek WY, Lee MA, Jung JW, Kim SY, Akiyama H, de Crombrugghe B, et al. Positive regulation of adult bone formation by osteoblast-specific transcription factor osterix. J Bone Miner Res. 2009;24(6):1055-65.
91. Lin GL, Hankenson KD. Integration of BMP, Wnt, and notch signaling pathways in osteoblast differentiation. J Cell Biochem. 2011;112(12):3491-501.
92. Celeste AJ, Iannazzi JA, Taylor RC, Hewick RM, Rosen V, Wang EA, et al. Identification of transforming growth factor beta family members present in bone-inductive protein purified from bovine bone. Proc Natl Acad Sci. 1990;87(24):9843-7.
93. Chen G, Deng C, Li Y-P. TGF-β and BMP signaling in osteoblast differentiation and bone formation. Int J Biol Sci. 2012;8(2):272.
94. Yoshida Y, Tanaka S, Umemori H, Minowa O, Usui M, Ikematsu N, et al. Negative regulation of BMP/Smad signaling by Tob in osteoblasts. Cell. 2000;103(7):1085-97.
95. Nishimura R, Kato Y, Chen D, Harris SE, Mundy GR, Yoneda T. Smad5 and DPC4 are key molecules in mediating BMP-2-induced osteoblastic

differentiation of the pluripotent mesenchymal precursor cell line C2C12. J Biol Chem. 1998;273(4):1872-9.
96. Kawabata M, Imamura T, Miyazono K. Signal transduction by bone morphogenetic proteins. Cytokine Growth Factor Rev. 1998;9(1):49-61.
97. Fujii M, Takeda K, Imamura T, Aoki H, Sampath TK, Enomoto S, et al. Roles of bone morphogenetic protein type I receptors and Smad proteins in osteoblast and chondroblast differentiation. Mol Biol Cell. 1999;10(11):3801-13.
98. Lamplot JD, Qin J, Nan G, Wang J, Liu X, Yin L, et al. BMP9 signaling in stem cell differentiation and osteogenesis. Am J Stem Cells. 2013;2(1):1.
99. Gori F, Thomas T, Hicok KC, Spelsberg TC, Riggs BL. Differentiation of human marrow stromal precursor cells: Bone morphogenetic protein-2 increases OSF2/CBFA1, enhances osteoblast commitment, and inhibits late adipocyte maturation. J Bone Miner Res. 1999;14(9):1522-35.
100. Franceschi RT, Xiao G. Regulation of the osteoblast-specific transcription factor, Runx2: Responsiveness to multiple signal transduction pathways. J Cell Biochem. 2003;88(3):446-54.
101. Zhang X, Yang M, Lin L, Chen P, Ma K, Zhou C, et al. Runx2 overexpression enhances osteoblastic differentiation and mineralization in adipose-derived stem cells in vitro and in vivo. Calcif Tissue Int. 2006;79(3):169-78.
102. Zhang F, Xu L, Xu L, Xu Q, Karsenty G, Chen CD. Histone demethylase JMJD3 is required for osteoblast differentiation in mice. Sci Rep. 2015;5:13418.
103. Ito H. Chemokines in mesenchymal stem cell therapy for bone repair: a novel concept of recruiting mesenchymal stem cells and the possible cell sources. Mod Rheumatol. 2011;21(2):113-21.
104. Hosogane N, Huang Z, Rawlins BA, Liu X, Boachie-Adjei O, Boskey AL, et al. Stromal derived factor-1 regulates bone morphogenetic protein 2-induced osteogenic differentiation of primary mesenchymal stem cells. Int J Biochem Cell Biol. 2010;42(7):1132-41.
105. Teicher BA, Fricker SP. CXCL12 (SDF-1)/CXCR4 pathway in cancer. Clin Cancer Res. 2010;16(11):2927-31.
106. Kitaori T, Ito H, Schwarz EM, Tsutsumi R, Yoshitomi H, Oishi S, et al. Stromal cell–derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model. Arthritis Rheumatol. 2009;60(3):813-23.
107. Muruganandan S, Roman A, Sinal C. Adipocyte differentiation of bone marrow-derived mesenchymal stem cells: cross talk with the osteoblastogenic program. Cell Mol Life Sci. 2009;66(2):236-53.
108. Davis L, Zur Nieden N. Mesodermal fate decisions of a stem cell: the Wnt switch. Cell Mol Life Sci. 2008;65(17):2658.
109. Huelsken J, Behrens J. The Wnt signalling pathway. J Cell Sci. 2002;115(21):3977-8.
110. Veeman MT, Axelrod JD, Moon RT. A second canon: functions and mechanisms of β-catenin-independent Wnt signaling. Dev Cell. 2003;5(3):367-77.
111. Rodríguez-Carballo E, Gámez B, Ventura F. p38 MAPK signaling in osteoblast differentiation. Front Cell Dev Biol. 2016;4:40.
112. Matsushita T, Chan YY, Kawanami A, Balmes G, Landreth GE, Murakami S. Extracellular signal-regulated kinase 1 (ERK1) and ERK2 play essential roles in osteoblast differentiation and in supporting osteoclastogenesis. Mol Cell Biol. 2009;29(21):5843-57.

113. Lai C-F, Chaudhary L, Fausto A, Halstead LR, Ory DS, Avioli LV, et al. Erk is essential for growth, differentiation, integrin expression, and cell function in human osteoblastic cells. J Biol Chem. 2001;276(17):14443-50.
114. Matsuguchi T, Chiba N, Bandow K, Kakimoto K, Masuda A, Ohnishi T. JNK activity is essential for Atf4 Expression and late-stage osteoblast differentiation. J Bone Miner Res. 2009;24(3):398-410.
115. Huang R, Yuan Y, Tu J, Zou G, Li Q. Opposing TNF-α/IL-1β-and BMP-2-activated MAPK signaling pathways converge on Runx2 to regulate BMP-2-induced osteoblastic differentiation. Cell Death Dis. 2014;5(4):e1187.
116. St-Jacques B, Hammerschmidt M, McMahon AP. Indian hedgehog signaling regulates proliferation and differentiation of chondrocytes and is essential for bone formation. Genes Dev. 1999;13(16):2072-86.
117. Mak KK, Chen M-H, Day TF, Chuang P-T, Yang Y. Wnt/β-catenin signaling interacts differentially with Ihh signaling in controlling endochondral bone and synovial joint formation. Development. 2006;133(18):3695-707.
118. Hu H, Hilton MJ, Tu X, Yu K, Ornitz DM, Long F. Sequential roles of Hedgehog and Wnt signaling in osteoblast development. Development. 2005;132(1):49-60.
119. Plaisant M, Fontaine C, Cousin W, Rochet N, Dani C, Peraldi P. Activation of hedgehog signaling inhibits osteoblast differentiation of human mesenchymal stem cells. Stem Cells. 2009;27(3):703-13.
120. Jimi E. Chapter four-The role of BMP signaling and NF-κB signaling on osteoblastic differentiation, cancer development, and vascular diseases—Is the Activation of NF-κB a friend or foe of BMP function? Vitam Horm. 2015;99:145-70.
121. Durandt C, Van Vollenstee FA, Dessels C, Kallmeyer K, De Villiers D, Murdoch C, et al. Novel flow cytometric approach for the detection of adipocyte subpopulations during adipogenesis. J Lipid Res. 2016;57(4):729-42.
122. Cruz MM, Lopes AB, Crisma AR, de Sá RC, Kuwabara WM, Curi R, et al. Palmitoleic acid (16: 1n7) increases oxygen consumption, fatty acid oxidation and ATP content in white adipocytes. Lipids Health Dis. 2018;17(1):55.
123. Liu Y, Wang N, Zhang S, Liang Q. Autophagy protects bone marrow mesenchymal stem cells from palmitate-induced apoptosis through the ROS-JNK/p38 MAPK signaling pathways. Mol Med Report. 2018.
124. Takada I, Kouzmenko AP, Kato S. PPAR-signaling crosstalk in mesenchymal stem cells. PPAR Res. 2010;2010.
125. Takada I, Kouzmenko AP, Kato S. Molecular switching of osteoblastogenesis versus adipogenesis: implications for targeted therapies. Expert Opin Ther Targets. 2009;13(5):593-603.
126. Hihi A, Michalik L, Wahli W. PPARs: transcriptional effectors of fatty acids and their derivatives. Cell Mol Life Sci. 2002;59(5):790-8.
127. Glass DA, Bialek P, Ahn JD, Starbuck M, Patel MS, Clevers H, et al. Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. Dev Cell. 2005;8(5):751-64.
128. Ross SE, Hemati N, Longo KA, Bennett CN, Lucas PC, Erickson RL, et al. Inhibition of adipogenesis by Wnt signaling. Science. 2000;289(5481):950-3.
129. Rosen ED, Spiegelman BM. Molecular regulation of adipogenesis. Annu Rev Cell Dev Biol. 2000;16(1):145-71.
130. Cristancho AG, Lazar MA. Forming functional fat: a growing understanding of adipocyte differentiation. Nat Rev Mol Cell Biol. 2011;12(11):722.

131. Desvergne B, Wahli W. Peroxisome proliferator-activated receptors: nuclear control of metabolism. Endocr Rev. 1999;20(5):649-88.
132. Lecka-Czernik B. PPARs in bone: the role in bone cell differentiation and regulation of energy metabolism. Curr Osteoporos Rep. 2010;8(2):84-90.
133. Yki-Järvinen H. Thiazolidinediones. New Engl J Med. 2004;351(11):1106-18.
134. Chan B, Gartland A, Wilson P, Buckley K, Dillon J, Fraser W, et al. PPAR agonists modulate human osteoclast formation and activity in vitro. Bone. 2007;40(1):149-59.
135. Tontonoz P, Hu E, Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. Cell. 1994;79(7):1147-56.
136. Marie PJ. Transcription factors controlling osteoblastogenesis. Arch Biochem Biophys. 2008;473(2):98-105.
137. Jeon MJ, Kim JA, Kwon SH, Kim SW, Park KS, Park S-W, et al. Activation of peroxisome proliferator-activated receptor-γ inhibits the Runx2-mediated transcription of osteocalcin in osteoblasts. J Biol Chem. 2003;278(26):23270-7.
138. Lecka-Czernik B, Moerman EJ, Grant DF, Lehmann JrM, Manolagas SC, Jilka RL. Divergent effects of selective peroxisome proliferator-activated receptor-γ2 ligands on adipocyte versus osteoblast differentiation. Endocrinol. 2002;143(6):2376-84.
139. Meunier P, Aaron J, Edouard C, VlGNON G. Osteoporosis and the replacement of cell populations of the marrow by adipose tissue: a quantitative study of 84 iliac bone biopsies. Clin Orthop Relat Res. 1971;80:147-54.
140. Diascro DD, Vogel RL, Johnson TE, Witherup KM, Pitzenberger SM, Rutledge SJ, et al. High fatty acid content in rabbit serum Is responsible for the differentiation of osteoblasts into adipocyte-like cells. J Bone Miner Res. 1998;13(1):96-106.
141. Hong J-H, Yaffe MB. TAZ: a β-catenin-like molecule that regulates mesenchymal stem cell differentiation. Cell cycle. 2006;5(2):176-9.
142. Roche HM. Unsaturated fatty acids. Proc Nutr Soc. 1999;58(2):397-401.
143. Dandekar A, Fisk H, McGranahan G, Uratsu S, Bains H, Leslie C, et al. Different genes for different folks in tree crops: what works and what does not. HortScience. 2002;37(2):281-6.
144. Mensink RP, Organization WH. Effects of saturated fatty acids on serum lipids and lipoproteins: a systematic review and regression analysis. 2016.
145. Calder PC. Functional roles of fatty acids and their effects on human health. JPEN J Parenter Enteral Nutr. 2015;39:18S-32S.
146. Risérus U. Fatty acids and insulin sensitivity. Curr Opin Clin Nutr Metab Care. 2008;11(2):100-5.
147. Forouhi NG, Koulman A, Sharp SJ, Imamura F, Kröger J, Schulze MB, et al. Differences in the prospective association between individual plasma phospholipid saturated fatty acids and incident type 2 diabetes: the EPIC-InterAct case-cohort study. Lancet Diabetes Endocrinol. 2014;2(10):810-8.
148. Holman RT. Measurement of polyunsaturated fatty acids. Methods Biochem Anal. 1957;4:99-138.
149. Shahidi F, Ambigaipalan P. Omega-3 Polyunsaturated Fatty Acids and Their Health Benefits. Annu Rev Food Sci Technol. 2018;9(1):345-81.
150. Yokoyama M, Origasa H, Matsuzaki M, Matsuzawa Y, Saito Y, Ishikawa Y, et al. Effects of eicosapentaenoic acid on major coronary events in

hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis. Lancet. 2007;369(9567):1090-8.
151. Endo J, Arita M. Cardioprotective mechanism of omega-3 polyunsaturated fatty acids. J Cardiol. 2016;67(1):22-7.
152. Innes JK, Calder PC. Omega-6 fatty acids and inflammation. Prostaglandins Leukot Essent Fatty Acids. 2018;132:41-8.
153. Simopoulos AP. The importance of the omega-6/omega-3 fatty acid ratio in cardiovascular disease and other chronic diseases. Exp Biol Med. 2008;233(6):674-88.
154. Larsson SC, Kumlin M, Ingelman-Sundberg M, Wolk A. Dietary long-chain n−3 fatty acids for the prevention of cancer: a review of potential mechanisms. Am J Clin Nutr. 2004;79(6):935-45.
155. Gil A. Polyunsaturated fatty acids and inflammatory diseases. Biomed Pharmacother. 2002;56(8):388-96.
156. Uauy R, Valenzuela A. Marine oils: the health benefits of n-3 fatty acids. Nutrition. 2000;16(7-8):680-4.
157. Kris-Etherton PM. Monounsaturated fatty acids and risk of cardiovascular disease. Circulation. 1999;100(11):1253-8.
158. Tarrago-Trani MT, Phillips KM, Lemar LE, Holden JM. New and existing oils and fats used in products with reduced trans-fatty acid content. J Am Diet Assoc. 2006;106(6):867-80.
159. Tosti V, Bertozzi B, Fontana L. Health benefits of the Mediterranean diet: metabolic and molecular mechanisms. J Gerontol A. 2017;73(3):318-26.
160. Schwingshackl L, Hoffmann G. Does a Mediterranean-type diet reduce cancer risk? Curr Nutr Rep. 2016;5(1):9-17.
161. Salas-Salvadó J, Guasch-Ferré M, Lee C-H, Estruch R, Clish CB, Ros E. Protective effects of the Mediterranean diet on type 2 diabetes and metabolic syndrome. J Nutr. 2015;146(4):920S-7S.
162. Petersson SD, Philippou E. Mediterranean diet, cognitive function, and dementia: a systematic review of the evidence. Adv Nutr. 2016;7(5):889-904.
163. Sansone A, Tolika E, Louka M, Sunda V, Deplano S, Melchiorre M, et al. Hexadecenoic fatty acid isomers in human blood lipids and their relevance for the interpretation of lipidomic profiles. PLoS One. 2016;11(4):e0152378.
164. Frigolet ME, Gutiérrez-Aguilar R. The Role of the Novel Lipokine Palmitoleic Acid in Health and Disease–. Adv Nutr. 2017;8(1):173S-81S.
165. Bolsoni-Lopes A, Festuccia WT, Farias TS, Chimin P, Torres-Leal FL, Derogis PB, et al. Palmitoleic acid (n-7) increases white adipocyte lipolysis and lipase content in a PPARα-dependent manner. Am J Physiol Endocrinol Metab. 2013;305(9):E1093-E102.
166. Mozaffarian D, Cao H, King IB, Lemaitre RN, Song X, Siscovick DS, et al. Circulating palmitoleic acid and risk of metabolic abnormalities and new-onset diabetes. Am J Clin Nutr. 2010;92(6):1350-8.
167. Nestel P, Clifton P, Noakes M. Effects of increasing dietary palmitoleic acid compared with palmitic and oleic acids on plasma lipids of hypercholesterolemic men. J Lipid Res. 1994;35(4):656-62.
168. Souza CO, Valenzuela CA, Baker EJ, Miles EA, Rosa Neto JC, Calder PC. Palmitoleic Acid has Stronger Anti-Inflammatory Potential in Human Endothelial Cells Compared to Oleic and Palmitic Acids. Mol Nutr Food Res. 2018;62(20):1800322.

169. Souza CO, Teixeira AA, Biondo LA, Silveira LS, Calder PC, Rosa Neto JC. Palmitoleic acid reduces the inflammation in LPS-stimulated macrophages by inhibition of NF κB, independently of PPAR s. Clin Exp Pharmacol Physiol. 2017;44(5):566-75.
170. Maslin K, Dennison E. Diet and Bone Health. Analysis in Nutrition Research: Elsevier; 2019. p. 337-54.
171. Albertazzi P, Coupland K. Polyunsaturated fatty acids. Is there a role in postmenopausal osteoporosis prevention? Maturitas. 2002;42(1):13-22.
172. Gillet C, Spruyt D, Rigutto S, Dalla Valle A, Berlier J, Louis C, et al. Oleate abrogates palmitate-induced lipotoxicity and proinflammatory response in human bone marrow-derived mesenchymal stem cells and osteoblastic cells. Endocrinol. 2015;156(11):4081-93.
173. Halade GV, Rahman MM, Williams PJ, Fernandes G. High fat diet-induced animal model of age-associated obesity and osteoporosis. J Nutr Biochem. 2010;21(12):1162-9.
174. Poulsen RC, Wolber FM, Moughan PJ, Kruger MC. Long chain polyunsaturated fatty acids alter membrane-bound RANK-L expression and osteoprotegerin secretion by MC3T3-E1 osteoblast-like cells. Prostaglandins Other Lipid Mediat. 2008;85(1-2):42-8.
175. Casado-Diaz A, Santiago-Mora R, Dorado G, Quesada-Gomez J. The omega-6 arachidonic fatty acid, but not the omega-3 fatty acids, inhibits osteoblastogenesis and induces adipogenesis of human mesenchymal stem cells: potential implication in osteoporosis. Osteoporosis Int. 2013;24(5):1647-61.
176. Choi B-Y, Eun J-S, Nepal M, Lee M-K, Bae T-S, Kim B-I, et al. Ethyl docosahexaenoate and its acidic form increase bone formation by induction of osteoblast differentiation and inhibition of osteoclastogenesis. Biomol Ther (Seoul). 2011;19(1):70-6.
177. Coetzee M, Haag M, Kruger MC. Effects of arachidonic acid and docosahexaenoic acid on differentiation and mineralization of MC3T3-E1 osteoblast-like cells. Cell Biochemistry and Function: Cellular biochemistry and its modulation by active agents or disease. 2009;27(1):3-11.
178. Drosatos-Tampakaki Z, Drosatos K, Siegelin Y, Gong S, Khan S, Van Dyke T, et al. Palmitic acid and DGAT1 deficiency enhance osteoclastogenesis, while oleic acid-induced triglyceride formation prevents it. J Bone Miner Res. 2014;29(5):1183-95.
179. Kyriakis JM, Avruch J. Mammalian MAPK signal transduction pathways activated by stress and inflammation: a 10-year update. Physiol Rev. 2012;92(2):689-737.
180. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science. 1995;270(5240):1326-31.
181. Fister S, Günthert AR, Aicher B, Paulini KW, Emons G, Gründker C. GnRH-II antagonists induce apoptosis in human endometrial, ovarian, and breast cancer cells via activation of stress-induced MAPKs p38 and JNK and proapoptotic protein Bax. Cancer Res. 2009;69(16):6473-81.
182. Tang C, Liang J, Qian J, Jin L, Du M, Li M, et al. Opposing role of JNK-p38 kinase and ERK1/2 in hydrogen peroxide-induced oxidative damage of human trophoblast-like JEG-3 cells. Int J Clin Exp Pathol. 2014;7(3):959.

183. Cornish J, MacGibbon A, Lin J-M, Watson M, Callon KE, Tong P, et al. Modulation of osteoclastogenesis by fatty acids. Endocrinol. 2008;149(11):5688-95.
184. Boeyens J, Deepak V, Chua W-H, Kruger M, Joubert A, Coetzee M. Effects of ω3-and ω6-polyunsaturated fatty acids on RANKL-induced osteoclast differentiation of RAW264. 7 cells: a comparative in vitro study. Nutrients. 2014;6(7):2584-601.
185. Rahman MM, Bhattacharya A, Fernandes G. Docosahexaenoic acid is more potent inhibitor of osteoclast differentiation in RAW 264.7 cells than eicosapentaenoic acid. J Cell Physiol. 2008;214(1):201-9.
186. Kasonga AE, Deepak V, Kruger MC, Coetzee M. Arachidonic acid and docosahexaenoic acid suppress osteoclast formation and activity in human CD14+ monocytes, in vitro. PLoS One. 2015;10(4):e0125145.
187. Kasonga A, Kruger MC, Coetzee M. Activation of PPARs Modulates Signalling Pathways and Expression of Regulatory Genes in Osteoclasts Derived from Human CD14+ Monocytes. Int J Mol Sci. 2019;20(7):1798.
188. Malmir H, Saneei P, Larijani B, Esmaillzadeh A. Adherence to Mediterranean diet in relation to bone mineral density and risk of fracture: a systematic review and meta-analysis of observational studies. Eur J Nutr. 2018:1-14.
189. Benetou V, Orfanos P, Feskanich D, Michaëlsson K, Pettersson-Kymmer U, Byberg L, et al. Mediterranean diet and hip fracture incidence among older adults: the CHANCES project. Osteoporosis Int. 2018;29(7):1591-9.
190. Anez-Bustillos L, Cowan E, Cubria MB, Villa-Camacho JC, Mohamadi A, Dao DT, et al. Effects of dietary omega-3 fatty acids on bones of healthy mice. Clin Nutr. 2018.
191. Song J, Jing Z, Hu W, Yu J, Cui X. α-Linolenic acid inhibits receptor activator of NF-κB ligand induced (RANKL-Induced) osteoclastogenesis and prevents inflammatory bone loss via downregulation of nuclear factor-KappaB-inducible nitric oxide synthases (NF-κB-iNOS) signaling pathways. Med Sci Monit. 2017;23:5056.
192. Lanham S, Roberts C, Hollingworth T, Sreekumar R, Elahi M, Cagampang F, et al. Maternal high-fat diet: effects on offspring bone structure. Osteoporosis Int. 2010;21(10):1703-14.
193. Mousavi SN, Koohdani F, Eslaminejad MB, Izadi P, Eshraghian M, Sayahpour FA, et al. Extra virgin olive oil in maternal diet increases osteogenic genes expression, but high amounts have deleterious effects on bones in mice offspring at adolescence. Iran J Basic Med Sci. 2016;19(12):1299.
194. Chen T-y, Zhang Z-m, Zheng X-c, Wang L, Huang M-j, Qin S, et al. Endogenous n-3 polyunsaturated fatty acids (PUFAs) mitigate ovariectomy-induced bone loss by attenuating bone marrow adipogenesis in FAT1 transgenic mice. Drug Des Devel Ther. 2013;7:545.
195. Poulsen R, Firth E, Rogers C, Moughan P, Kruger M. Specific effects of γ-Linolenic, eicosapentaenoic, and docosahexaenoic ethyl esters on bone post-ovariectomy in rats. Calcif Tissue Int. 2007;81(6):459-71.
196. Li Y, Seifert MF, Lim S-Y, Salem N, Watkins BA. Bone mineral content is positively correlated to n-3 fatty acids in the femur of growing rats. Br J Nutr. 2010;104(5):674-85.
197. Corwin RL, Hartman TJ, Maczuga SA, Graubard BI. Dietary saturated fat intake is inversely associated with bone density in humans: analysis of NHANES III. J Nutr. 2006;136(1):159-65.

198. Rajaram S, Yip E, Reghunathan R, Mohan S, Sabaté J. Effect of Altering Dietary n-6: n-3 Polyunsaturated Fatty Acid Ratio with Plant and Marine-Based Supplement on Biomarkers of Bone Turnover in Healthy Adults. Nutrients. 2017;9(10):1162.
199. Griel AE, Kris-Etherton PM, Hilpert KF, Zhao G, West SG, Corwin RL. An increase in dietary n-3 fatty acids decreases a marker of bone resorption in humans. Nutr J. 2007;6(1):2.
200. Weiss LA, Barrett-Connor E, von Mühlen D. Ratio of n–6 to n–3 fatty acids and bone mineral density in older adults: the Rancho Bernardo Study. Am J Clin Nutr. 2005;81(4):934-8.
201. Vinding RK, Stokholm J, Sevelsted A, Sejersen T, Chawes BL, Bønnelykke K, et al. Effect of fish oil supplementation in pregnancy on bone, lean, and fat mass at six years: randomised clinical trial. BMJ. 2018;362:k3312.
202. Virtanen JK, Mozaffarian D, Cauley JA, Mukamal KJ, Robbins J, Siscovick DS. Fish consumption, bone mineral density, and risk of hip fracture among older adults: the cardiovascular health study. J Bone Miner Res. 2010;25(9):1972-9.
203. Muraki S, Yamamoto S, Ishibashi H, Oka H, Yoshimura N, Kawaguchi H, et al. Diet and lifestyle associated with increased bone mineral density: cross-sectional study of Japanese elderly women at an osteoporosis outpatient clinic. J Orthop Sci. 2007;12(4):317-20.
204. Farina EK, Kiel DP, Roubenoff R, Schaefer EJ, Cupples LA, Tucker KL. Protective effects of fish intake and interactive effects of long-chain polyunsaturated fatty acid intakes on hip bone mineral density in older adults: the Framingham Osteoporosis Study. Am J Clin Nutr. 2011;93(5):1142-51.
205. Fonolla-Joya J, Reyes-García R, García-Martín A, López-Huertas E, Muñoz-Torres M. Daily intake of milk enriched with n-3 fatty acids, oleic acid, and calcium improves metabolic and bone biomarkers in postmenopausal women. J Am Coll Nutr. 2016;35(6):529-36.
206. Harris M, Farrell V, Houtkooper L, Going S, Lohman T. Associations of polyunsaturated fatty acid intake with bone mineral density in postmenopausal women. J Osteoporos. 2015;2015.
207. Feskanich D, Willett WC, Colditz GA. Calcium, vitamin D, milk consumption, and hip fractures: a prospective study among postmenopausal women. Am J Clin Nutr. 2003;77(2):504-11.
208. Zalloua PA, Hsu Y-H, Terwedow H, Zang T, Wu D, Tang G, et al. Impact of seafood and fruit consumption on bone mineral density. Maturitas. 2007;56(1):1-11.
209. Chen Y-M, Ho S, Lam S. Higher sea fish intake is associated with greater bone mass and lower osteoporosis risk in postmenopausal Chinese women. Osteoporosis Int. 2010;21(6):939-46.
210. Virtanen JK, Mozaffarian D, Willett WC, Feskanich D. Dietary intake of polyunsaturated fatty acids and risk of hip fracture in men and women. Osteoporosis Int. 2012;23(11):2615-24.
211. Calderon-Garcia J, Moran J, Roncero-Martin R, Rey-Sanchez P, Rodriguez-Velasco F, Pedrera-Zamorano J. Dietary habits, nutrients and bone mass in Spanish premenopausal women: the contribution of fish to better bone health. Nutrients. 2012;5(1):10-22.
212. Fujiwara S, Kasagi F, Yamada M, Kodama K. Risk factors for hip fracture in a Japanese cohort. J Bone Miner Res. 1997;12(7):998-1004.

213. Ambele MA, Dessels C, Durandt C, Pepper MS. Genome-wide analysis of gene expression during adipogenesis in human adipose-derived stromal cells reveals novel patterns of gene expression during adipocyte differentiation. Stem Cell Res. 2016;16(3):725-34.
214. Pautke C, Schieker M, Tischer T, Kolk A, Neth P, Mutschler W, et al. Characterization of osteosarcoma cell lines MG-63, Saos-2 and U-2 OS in comparison to human osteoblasts. Anticancer Res. 2004;24(6):3743-8.
215. Staehlke S, Rebl H, Nebe B. Phenotypic stability of the human MG-63 osteoblastic cell line at different passages. Cell Biol Int. 2019;43(1):22-32.
216. Heremans H, Billiau A, Cassiman J-J, Mulier J, De Somer P. In vitro cultivation of human tumor tissues II. Morphological and virological characterization of three cell lines. Oncology. 1978;35(6):246-52.
217. Louis KS, Siegel AC. Cell viability analysis using trypan blue: manual and automated methods. Mammalian Cell Viability: Springer; 2011. p. 7-12.
218. Rampersad SN. Multiple applications of Alamar Blue as an indicator of metabolic function and cellular health in cell viability bioassays. Sensors. 2012;12(9):12347-60.
219. Al-Nasiry S, Geusens N, Hanssens M, Luyten C, Pijnenborg R. The use of Alamar Blue assay for quantitative analysis of viability, migration and invasion of choriocarcinoma cells. Hum Reprod. 2007;22(5):1304-9.
220. Song IH, Caplan AI, Dennis JE. In vitro dexamethasone pretreatment enhances bone formation of human mesenchymal stem cells in vivo. J Orth Res. 2009;27(7):916-21.
221. Song IH, Caplan AI, Dennis JE. Dexamethasone inhibition of confluence-induced apoptosis in human mesenchymal stem cells. J Orth Res. 2009;27(2):216-21.
222. Wang H, Pang B, Li Y, Zhu D, Pang T, Liu Y. Dexamethasone has variable effects on mesenchymal stromal cells. Cytotherapy. 2012;14(4):423-30.
223. Langenbach F, Handschel J. Effects of dexamethasone, ascorbic acid and β-glycerophosphate on the osteogenic differentiation of stem cells in vitro. Stem Cell Res Ther. 2013;4(5):117.
224. Golub EE, Boesze-Battaglia K. The role of alkaline phosphatase in mineralization. Curr Opin Orthop. 2007;18(5):444-8.
225. Shamsi M, Karimi M, Ghollasi M, Nezafati N, Shahrousvand M, Kamali M, et al. In vitro proliferation and differentiation of human bone marrow mesenchymal stem cells into osteoblasts on nanocomposite scaffolds based on bioactive glass (64SiO2-31CaO-5P2O5)-poly-l-lactic acid nanofibers fabricated by electrospinning method. Mater Sci Eng C. 2017;78:114-23.
226. Gregory CA, Gunn WG, Peister A, Prockop DJ. An Alizarin red-based assay of mineralization by adherent cells in culture: comparison with cetylpyridinium chloride extraction. Anal Biochem. 2004;329(1):77-84.
227. Visagie A, Kasonga A, Deepak V, Moosa S, Marais S, Kruger M, et al. Commercial honeybush (cyclopia spp.) tea extract inhibits osteoclast formation and bone resorption in RAW264. 7 murine macrophages—an in vitro study. Int J Env Res Public Health. 2015;12(11):13779-93.
228. Lorenz TC. Polymerase chain reaction: basic protocol plus troubleshooting and optimization strategies. J Vis Exp. 2012(63).
229. Mahmood T, Yang P-C. Western blot: technique, theory, and trouble shooting. N Am J Med Sci. 2012;4(9):429.

230. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012;9(7):671.
231. Aldridge A, Kouroupis D, Churchman S, English A, Ingham E, Jones E. Assay validation for the assessment of adipogenesis of multipotential stromal cells-a direct comparison of four different methods. Cytotherapy. 2013;15(1):89-101.
232. Sekiya I, Larson BL, Smith JR, Pochampally R, Cui JG, Prockop DJ. Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. Stem Cells. 2002;20(6):530-41.
233. Martella E, Bellotti C, Dozza B, Perrone S, Donati D, Lucarelli E. Secreted adiponectin as a marker to evaluate in vitro the adipogenic differentiation of human mesenchymal stromal cells. Cytotherapy. 2014;16(11):1476-85.
234. Welters HJ, Diakogiannaki E, Mordue JM, Tadayyon M, Smith SA, Morgan NG. Differential protective effects of palmitoleic acid and cAMP on caspase activation and cell viability in pancreatic β-cells exposed to palmitate. Apoptosis. 2006;11(7):1231-8.
235. Sabokbar A, Millett P, Myer B, Rushton N. A rapid, quantitative assay for measuring alkaline phosphatase activity in osteoblastic cells in vitro. Bone Miner. 1994;27(1):57-67.
236. James AW, Xu Y, Wang R, Longaker MT. Proliferation, osteogenic differentiation, and FGF-2 modulation of posterofrontal/sagittal suture–derived mesenchymal cells in vitro. Plast Reconstr Surg. 2008;122(1):53-63.
237. Weinzierl K, Hemprich A, Frerich B. Bone engineering with adipose tisssue derived stromal cells. J Craniomaxillofac Surg. 2006;34(8):466-71.
238. Czekanska EM, Stoddart MJ, Ralphs JR, Richards R, Hayes J. A phenotypic comparison of osteoblast cell lines versus human primary osteoblasts for biomaterials testing. J Biomed Mater Res A. 2014;102(8):2636-43.
239. Hale L, Ma Y, Santerre R. Semi-quantitative fluorescence analysis of calcein binding as a measurement of in vitro mineralization. Calcif Tissue Int. 2000;67(1):80-4.
240. Lievremont M, Potus J, Guillou B. Use of alizarin red S for histochemical staining of Ca2+ in the mouse; some parameters of the chemical reaction in vitro. Cells Tissues Organs. 1982;114(3):268-80.
241. Anselme K, Broux O, Noel B, Bouxin B, Bascoulergue G, Dudermel A-F, et al. In vitro control of human bone marrow stromal cells for bone tissue engineering. Tissue Eng. 2002;8(6):941-53.
242. Li X, Yao J, Wu L, Jing W, Tang W, Lin Y, et al. Osteogenic induction of adipose-derived stromal cells: not a requirement for bone formation in vivo. Artif Organs. 2010;34(1):46-54.
243. Di Battista JA, Shebaby W, Kizilay O, Hamade E, Merhi RA, Mebarek S, et al. Proliferation and differentiation of human adipose-derived mesenchymal stem cells (ASCs) into osteoblastic lineage are passage dependent. Inflammation Res. 2014;63(11):907-17.
244. Czekanska E, Stoddart M, Richards R, Hayes J. In search of an osteoblast cell model for in vitro research. Eur Cell Mater. 2012;24(4):1-17.
245. Wang D, Cui L, Chang X, Guan D. Biosynthesis and characterization of zinc oxide nanoparticles from Artemisia annua and investigate their effect on proliferation, osteogenic differentiation and mineralization in human osteoblast-like MG-63 Cells. J Photochem Photobiol B. 2019:111652.

246. Miron R, Zhang Y. Osteoinduction: a review of old concepts with new standards. J Dent Res. 2012;91(8):736-44.
247. Liu G, Zhou H, Li Y, Li G, Cui L, Liu W, et al. Evaluation of the viability and osteogenic differentiation of cryopreserved human adipose-derived stem cells. Cryobiology. 2008;57(1):18-24.
248. Egusa H, Iida K, Kobayashi M, Lin TY, Zhu M, Zuk PA, et al. Downregulation of extracellular matrix-related gene clusters during osteogenic differentiation of human bone marrow-and adipose tissue-derived stromal cells. Tissue Eng. 2007;13(10):2589-600.
249. Ye C, Chen M, Chen E, Li W, Wang S, Ding Q, et al. Knockdown of FOXA2 enhances the osteogenic differentiation of bone marrow-derived mesenchymal stem cells partly via activation of the ERK signalling pathway. Cell Death Dis. 2018;9(8):836.
250. Thouverey C, Caverzasio J. Focus on the p38 MAPK signaling pathway in bone development and maintenance. BoneKEy reports. 2015;4.
251. Hu K-H, Li W-X, Sun M-Y, Zhang S-B, Fan C-X, Wu Q, et al. Cadmium induced apoptosis in MG63 cells by increasing ROS, activation of p38 MAPK and inhibition of ERK 1/2 pathways. Cell Physiol Biochem. 2015;36(2):642-54.
252. Akune T, Ohba S, Kamekura S, Yamaguchi M, Chung U-i, Kubota N, et al. PPAR γ insufficiency enhances osteogenesis through osteoblast formation from bone marrow progenitors. J Clin Invest. 2004;113(6):846-55.
253. Lansdown A, Ludgate M, Rees A. Metabolic syndrome: is the preadipocyte to blame? Clin Endocrinol (Oxf). 2012;76(1):19.
254. Abdelmagid SA, Clarke SE, Nielsen DE, Badawi A, El-Sohemy A, Mutch DM, et al. Comprehensive profiling of plasma fatty acid concentrations in young healthy Canadian adults. PLoS One. 2015;10(2):e0116195.
255. Viegas M, Dias J, Cancela M, Laizé V. Polyunsaturated fatty acids regulate cell proliferation, extracellular matrix mineralization and gene expression in a gilthead seabream skeletal cell line. J Appl Ichthyol. 2012;28(3):427-32.
256. Minetti M, Bernardini G, Biazzo M, Gutierrez G, Geminiani M, Petrucci T, et al. Padina pavonica Extract Promotes In Vitro Differentiation and Functionality of Human Primary Osteoblasts. Mar Drugs. 2019;17(8):473.

APPENDIX A

Ethical approval

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

17 May 2019

Approval Certificate
Amendment

Ethics Reference No.: 386/2018
Title: Old: In vitro effects of palmitoleic acid on osteoblast differentiation in SaOS-2 osteosarcoma cells and human adipose-derived stromal cells.

New: In vitro effects of palmitoleic acid on osteoblast differentiation in MG-63 osteosarcoma cells and human adipose-derived stromal cells

Dear Ms K Howard

The **Amendment** as supported by documents received between 2019-05-07 and 2019-05-17 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-05-15.

Please note the following about your ethics approval:

- Please remember to use your protocol number (386/2018) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
Deputy Chairperson of the Faculty of Health Sciences Research Ethics Committee, University of Pretoria

The Faculty of Health Sciences Research Ethics Committee complies with the SA National Act 61 of 2003 as it pertains to health research and the United States Code of Federal Regulations Title 45 and 46. This committee abides by the ethical norms and principles for research, established by the Declaration of Helsinki, the South African Medical Research Council Guidelines as well as the Guidelines for Ethical Research: Principles Structures and Processes, Second Edition 2015 (Department of Health).

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Health Sciences

The Research Ethics Committee, Faculty Health Sciences, University of Pretoria complies with ICH-GCP guidelines and has US Federal wide Assurance.

- FWA 00002567, Approved dd 22 May 2002 and Expires 03/20/2022.
- IRB 0000 2235 IORG0001762 Approved dd 22/04/2014 and Expires 03/14/2020.

9 October 2019

Approval Certificate
Annual Renewal

Ethics Reference No.: 386/2018
Title: In vitro effects of palmitoleic acid on osteoblast differentiation in MG-63 osteosarcoma cells and human adipose-derived stromal cells

Dear Ms K Howard

The **Annual Renewal** as supported by documents received between 2019-09-03 and 2019-10-09 for your research, was approved by the Faculty of Health Sciences Research Ethics Committee on its quorate meeting of 2019-10-09.

Please note the following about your ethics approval:

- Renewal of ethics approval is valid for 1 year, subsequent annual renewal will become due on 2020-10-09.
- Please remember to use your protocol number (386/2018) on any documents or correspondence with the Research Ethics Committee regarding your research.
- Please note that the Research Ethics Committee may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.

Yours sincerely

Dr R Sommers
MBChB MMed (Int) MPharmMed PhD
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APPENDIX B

Statistical clearance

Date: 14 / 5 /20 19

LETTER OF CLEARANCE FROM THE BIOSTATISTICIAN

This letter is to confirm that,

Name(s): Ms KAYLA HOWARD

from the University of PRETORIA

discussed with me the study titled In vitro effects of palmitoleic acid on osteoblast differentiation in MG-63 osteosarcoma cells and human adipose-derived stromal cells

I hereby confirm that I am aware of the project and also undertake to assist, if possible, with the Statistical analysis of the data generated from the project.

The analytical tool(s) that will be used is (are) Descriptive Statistics by cell line and concentration, two-way ANOVA, mixed effects maximum likelihood regression. See also refer to attached section from protocol

to achieve the objective(s) of the study.

Name: **PJ Becker** **(Tel:** 012-319-2203)

Signature _____

Research Office,

Faculty of Health Sciences, UP

BIOSTATISTICS
Faculty of Health Sciences
Research Office
2019 -05- 14
UNIVERSITY OF PRETORIA

APPENDIX C

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INTRODUCTION

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